식물조직배양학회지 제24권 제5호
Korean J. Plant Tissue Culture
Vol. 24, No. 5, 273~277 (1997)

Induction of Sesquiterpene Cyclase During Integrated Extraction of Sesquiterpenes from Hairy Root Cultures of *Hyoscyamus muticus*

BACK, Kyoungwhan · SHIN, Dong Hyun* · KIM, Kil Ung · De HAAS, Cynthia R.[1] · CHAPPELL, Joseph[2] · CURTIS, Wayne R.[1]

Department of Agronomy, Kyungpook National University, Taegu, 702-701, Korea:
[1]Department of Chemical Engineering, The Pennsylvania State University, University Park, PA16802, USA: and
[2]Department of Agronomy, University of Kentucky, Lexington, KY40546-0091, USA. *Corresponding author.

*Hyoscyamus muticus*의 모상근배양으로부터 Sesquiterpene 화합물의 Intergration 추출시 Sesquiterpene Cyclase의 유도

백경환 · 신동현* · 김길웅 · De HAAS Cynthia R.[1] · CHAPPELL Joseph[2] · CURTIS Wayne R.[1]

경북대학교 농과대학 농학과, [1]미국 펜실바니아 주립대학교 화학공학과, [2]미국 켄터키 주립대학교 농학과

The sesquiterpene cyclase (SC) was induced and its products were accumulated in the culture media of *Hyoscyamus muticus* hairy roots by addition of *Rhizoctonia solani* extracts. The cumulative production of solavetivone was nearly doubled by integrated extraction of the products from the media during the 24 h accumulation period. Western blots with monoclonal antibodies against SC show that the enzyme levels are the same for both extracted and non-extracted cultures. SC activities measured *in vitro* with radioactive substrate are not significantly different. These results suggest that productivity is controlled by substrate availability within the terpenoid pathway, and feedback regulation precedes the branch-point enzyme sesquiterpene cyclase.

Key words: product inhibition, feedback regulation, integrated extraction, fungal elicitation, hairy root culture

The term "integrated extraction" refers to a variety of techniques where metabolic products are removed from the culture medium as they are produced. This can be accomplished either with solid adsorbents, or biocompatible solvents. The production rate of secondary metabolites can often be increased by integrated extraction. Such enhancements have been observed in essentially all culture systems, both prokaryotic and eukaryotic (van der Wielen and Luyben, 1992). Simply increasing the sink capacity does not provide much insight into the biochemical basis for enhanced production. In fact, increased production can result in the absence of enhanced metabolism either by preventing degradation of the product or simply redistributing metabolites such as facilitating release from the cells.

In cases where integrated extraction does result in increased biosynthesis, there are numerous explanations. Biosynthesis could be regulated by altered enzyme levels or enzyme activity. A large number of kinetic models exist which account for product inhibition including reversibility and competitive / non-competitive binding. Unfortunately, the metabolic pathways for most secondary metabolites are not known: therefore, the metabolic basis for feedback inhibition is not amenable to examination. In cases where specific enzymes can be identified, rigorous studies of *in vitro* enzyme activities are very difficult to conduct because there is little control over substrate or product levels. Also, secondary metabolites are usually present in low concentrations which introduces analytical difficulties for conducting kinetic studies. There is added complexity in whole cell systems because the levels of enzyme can be controlled either through transcriptional control of mRNA levels, translationally during the process of protein formation, or by enzyme turnover. In addition to enzyme controlled production, the substrate available could also limit production.

The model system chosen for the present work is the induction of sesquiterpenes from cultured plant roots of *Hyoscyamus muticus* exposed to fungal elicitor. This experimental system has several advantages for examining the mechanistic basis of productivity enhancement.

1) Sesquiterpene formation appears to be feedback inhibited: It has been demonstrated that sesquiterpene formation can be enhanced by integrated extraction (Corry et al., 1993) and by reducing the media concentration by dilution (Flores and Curtis, 1992).
2) A key induced branch-point enzyme has been identified and characterized: The branch-point enzyme which is the committed step towards sesquiterpene formation has been extensively characterized (Vogeli and Chappell, 1988, 1990). As a result of these studies, antibodies are available to detect enzyme levels, and radioactive precursors have been generated to quantitatively determine *in vitro* enzyme activity.

A schematic of the pathway is shown in Figure 1. Under non-elicited growth conditions, acetyl-CoA is a precursor to sterol synthesis which among other roles is used for the construction of cell membranes. In response to elicitor treatment, the enzyme sesquiterpene cyclase is induced resulting in an accumulation of sesquiterpenes (Vogeli and Chappell, 1988). Cyclase induction is accompanied by suppression of sterol synthesis through coordinated inhibition of squalene synthetase (Threlfall and Whitehead, 1988). The objective of this study was to probe more deeply into the biochemical / molecular mechanisms which may be responsible for the observed feedback inhibition of phytoalexin formation when root tissues are exposed to an extract of a root pathogenic fungi.

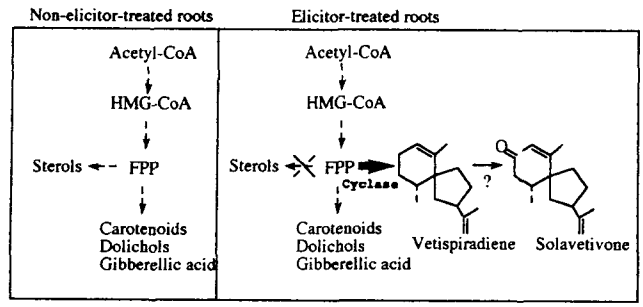

Figure 1. Schematic of the biosynthetic pathway leading to the production of the sesquiterpene solavetivone. The branch-point enzyme, sesquiterpene cyclase is induced upon addition of fungal elicitor.

MATERIALS AND METHODS

Root Culture, Elicitation, and Integrated Extraction

Procedures and conditions used for root culture, fungal elicitation, and integrated extraction are described in more detail elsewhere (Corry et al., 1993). The hairy root cultures were obtained by transformation of *Hyoscyamus muticus* with *Agrobacterium rhizogenes*. Sesquiterpenes are induced for 24 h by addition of 3 mL of fungal extract of *Rhizoctonia solani* which was from the same elicitor preparation as previous work (Corry et al., 1993). The carbohydrate content of the crude elicitor was measured at 4.76 mg glucose equivalents per liter by the anthrone method (Dische, 1961). Integrated extraction was carried out during the 24 h induction period on a gyratory shaker table with media moved to and from the extractor using peristaltic pumps. The 125 mL Erlenmeyer flasks subjected to integrated extraction contained 68 mL of fresh media and 25 mL of hexane.

Analysis of Sesquiterpenes

Solavetivone was recovered from the media and hexane phase by chloroform extraction and evaporation as described previously (Corry et al., 1993). HPLC procedures were carried out as described in this previous work: however, calculation of solavetivone levels was based on calibration curves generated with purified solavetivone as described more recently (Reddy et al., 1993).

Cyclase Assay

Roots (fr wt 0.5 g) were homogenized in a mortar and pestle as described previously (Vogeli and Chappell, 1988). The homogenate was centrifuged in an Eppendorf centrifuge for 10 min at 12,000 g at 4°C. Five microliter of supernatant was incubated in a total volume of 50 μL containing 200 mM Tris/HCl (pH 7.5), 40 mM $MgCl_2$, 3 nM of [1-^{3}H]FPP. After incubation for 30 min at 35°C, the reaction mixture was extracted with 150 μL of n-hexane, and an aliquot of the hexane phase reaction mixed with 20 mg of silica powder to remove any contaminating FPP or farnesol generated by phosphatase activity. The radioactivity remaining in the silica scrubbed hexane phase (representing the sesquiterpene product) was determined by scintillation counting. Protein concentration was estimated using the BioRad Protein Assay

and sesquiterpene cyclase activity expressed as nmol of sesquiterpene product / mg protein · h.

Western Blot Analysis

The soluble proteins (100 μg) were separated by SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membrane and immunodetected as described previously (Vogeli and Chappell, 1990).

RESULTS AND DISCUSSION

Cultures which were subjected to continuous extraction of the media phase following fungal elicitation produced 62% more sesquiterpenes than the non-extracted controls. The sesquiterpenes were concentrated in the hexane phase nearly 5 fold thereby reducing the concentration in the media by about 50%. As shown in Figure 2, these results are very consistent with previously published results (Corry et al., 1993). Enhancement of elicitor induced secondary metabolism by integrated extraction has been observed with other plant tissue culture systems as well. Addition of solid-phase adsorbents to *Catharanthus roseus* cultures increased ajmalicine production nearly 5 fold over elicitation without integrated extraction (Asada and Shuler, 1989). Elicitation in 2-phase culture of *Eschscholtzia californica* increased benzophenanthridine alkaloids 9 fold (Brodelius and Pederson, 1993: Byun et al., 1990).

Because increased production can result reduced product degradation, sesquiterpene stability was examined to assure that the increase in sesquiterpene production was to due to increased biosynthesis. It was found that the sesquiterpene levels were stable for over a week in hexane, culture media, or culture media in the presence of plant tissue. In addition, the levels of sesquiterpenes within the root tissue were negligible: therefore, the observed enhancement was not due to product release. These results indicate that the increase in production is due primarily to increased *de novo* synthesis of the sesquiterpenes.

Western blots of the induced root tissues at the time of media harvest are shown in Figure 3 (note that non-elicited controls show no enzyme protein). These blots indicate that there is not a significant difference in the levels of enzyme for extracted and non-extracted conditions. Since the levels of the enzyme are the same, this suggests that neither transcription of the cyclase gene nor translation of the cyclase messenger

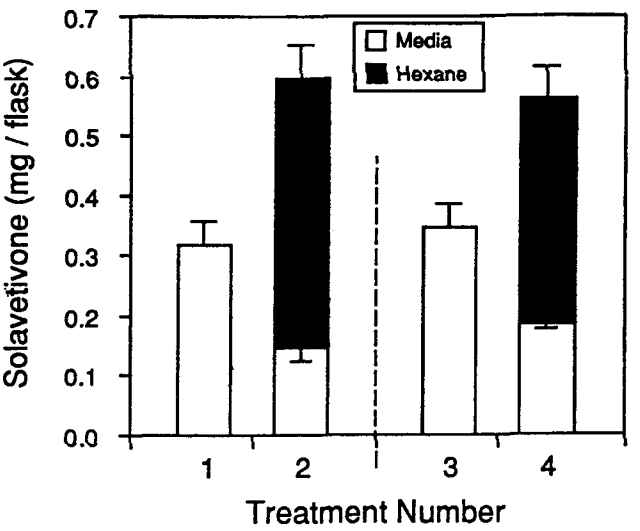

Figure 2. Cumulative production of solavetivone in hairy root cultures of *Hyoscyamus muticus* after 24 h exposure to fungal elicitor. Treatments 2 and 4 were subjected to integrated extraction by recirculating media through hexane in an external liquid-liquid extractor. Treatments 1 and 2 are the data previously published by Corry et al., (1993), recalculated according to purified sesquiterpene standards (Reddy et al., 1993). treatments 3 and 4 are a replicated experiment of 1 and 2 to assess the amount and activity of induced sesquiterpene cyclase enzyme.

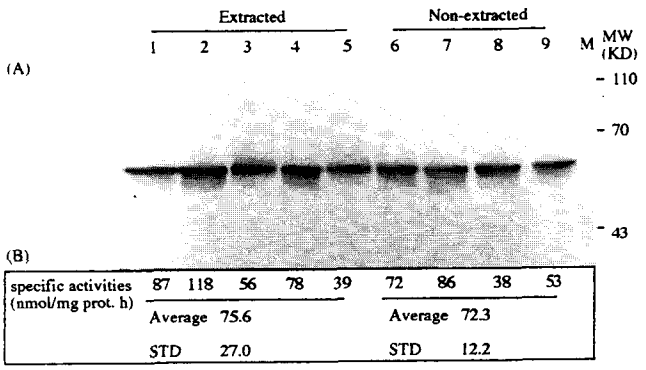

Figure 3. Immunoblot of sesquiterpene cyclase (A) and *in vitro* cyclase activity (B) for hairy root cultures of *Hyoscyamus muticus* with and without integrated extraction. M: molecular weight standards, Lanes 6-9 are replicated flasks elicited without extraction (treatment 3, Fig. 2): lanes 1-5 were subjected to hexane extraction during the 24-hour period following elicitation (treatment 4, Fig. 2). 100 μg protein was loaded per lane.

RNA are feedback controlled by the concentration of sesquiterpenes in media. Experiments to detect the cyclase mRNA levels (RNA blots) could have confirmed similar transcript levels: however, this was not persued since the

results would not be meaningful in the context of regulatory control of product formation.

Enzymes may remain immunologically detectable even if they are catalytically deactivated. The enzyme activities of extracted and non-extracted cultures were tested *in vitro* by examining the rate of conversion of radioactively labeled sesquiterpene substrate. Sesquiterpene cyclase enzyme activities were not significantly different based on a 95% "T" test: non-extracted, 72±12 nmol / mg protein, extracted: 76±27 nmol / mg protein. This demonstrates that both the extracted and non-extracted cultures have comparable catalytic capacity, and feedback inhibition does not enhance turnover of this branch-point enzyme. Whether sesquiterpene cyclase is inhibited *in vivo* could not be addressed. Solavetivone accumulates extracellularly, and is undetectable within the cell: therefore, if there is a direct interaction of the product and the enzyme, it must be taking place at concentration below our analytical detection limit. Direct addition to the *in vitro* assay is problematic because once solavetivone is extracted into organic solvents, it is difficult to redissolve it in aqueous solution without the aid of a co-solvent. Such co-solvents are known to interfere with the *in vitro* assay. Despite the limitations in probing *in vivo* enzyme activity, the combination of comparable enzyme protein levels and *in vitro* activities suggests that the mechanism of feedback inhibition is mediated through substrate limitation.

There is considerable evidence which suggests that substrate limitation may be common for secondary metabolic pathways. The ability to enhance secondary metabolite synthesis rates by precursor feeding is rather direct evidence that a pathway is substrate limited. For example, feeding of cholesterol to *Holarrhena antidysenterica* cultures resulted in greater than 3-fold enhancement in steroidal alkaloids (Panda et al., 1992). Addition of cinnamic acid increased vanillic acid formation, as did inhibition of ligneous material formation which competes for these precursors (Funk and Brodelius, 1990). Unfortunately, integrated extraction and precursor feeding are difficult to implement simultaneously because this requires an extraction phase which has a high affinity for the product, but not the precursor. Since product and precursor are chemically related by definition, achieving such a selectivity may not be possible. In this case of terpenoid metabolism, a logical precursor candidate might be mevalonate. We hope to persue this in the near future by carrying out the required measurements of tissue assimilation, stability and extraction phase distribution coefficients.

Another observation that is suggestive of substrate limited secondary metabolism is the ability to conduct biotransformation with exogenous substrates (Inomata et al., 1991: Suga et al., 1988). This indicates that there is considerable 'excess' enzymatic capacity which can be recruited to conduct alternate synthesis. Finally, there is a long-standing observation of a general competition between primary and secondary metabolism which is referred to as the 'inverse relationship' between primary and secondary metabolism. In general, rapidly growing cultures have lower secondary metabolite levels, and reduction in growth rate by nutrient limitation enhances secondary metabolite formation (Knobloch and Berlin, 1980). Such an inverse relationship would be expected for constitutive pathways where there is a competition for available resources. What we have shown here is that substrate limitation may also be playing an important role in regulation of the induced sesquiterpene pathway as well - despite the observation by others that competing pathways are down - regulated during the induction process (Threlfall and Whitehead, 1988). Future studies are being directed towards identifying those enzymes or secondary messengers which modulate resource allocation.

The observation that feedback inhibition does not appear to alter enzyme levels or activities has important implications to potential strategies for increasing secondary metabolism. In this experimental system, increasing the expression of a critical induced branch-point enzyme would not likely result in increased product formation. By analogy, ambitious attempts to clone and express genes in secondary metabolite pathways may fail to enhance culture productivities if the regulatory mechanisms which control resource allocation are not understood. The likelihood of substrate limitation has important implications to understanding the kinetics of secondary metabolite formation as well. Most enzyme kinetic models such as the rapid equilibrium approach of Michealis-Menten (Michaelis and Menten, 1913), or the pseudo-steady state approximation of Briggs-Haldane (Briggs and Haldane 1925), start with the assumption that substrate levels are in great excess of enzyme levels (Dixon and Wedd, 1979). The validation of these models has relied primarily on enzymes involved in primary metabolism - for which the assumption seems very reasonable (Newsholme and Start, 1973). Such an assumption is not necessarily valid for secondary metabolism and should be investigated throughly before extensive modeling and predictions are made with little experimental basis.

적 요

Rhizoctonia solani 추출물(elicitor)을 *Hyoscyamus muticus* 모상근배양에 처리하였을 때 sesquiterpene cyclase (SC)가 유도됨과 동시에 sesquiterpene 화합물이 합성되어 배양액속에 축적하는 것으로 나타났다. 24시간 배양기간 동안 배양액으로부터 sesquiterpene을 추출·분리하여 배양할 경우, 추출·분리하지 않은 모상근배양보다 거의 두배의 함량이 생성되었다. Cyclase monoclonal antibody을 이용하여 immunoblot을 시도한 결과, 추출한 모상근배양과 추출하지 않은 모상근배양에서의 SC 절대량은 동일한 것으로 나타났으며, 효소활성도 현저한 차이가 없는 것으로 측정되었다. 이러한 결과는 terpenoid pathway에서 sesquiterpene 생합성이 이용가능한 기질의 량에 의해 조절되는 것으로 사료되며, feedback 조절은 sesquiterpene cyclase 효소에 앞서 일어나는 것으로 추정된다.

ACKNOWLEDGEMENTS - We would like to acknowledge the assistance of Bill Reed in setting up the integrated extraction apparatus, and Ramakrishna Reddy for sesquiterpene analysis, and solavetivone stability studies. We also acknowledge the financial support of the National Science Foundation, a NSF Young Investigator Award to W.C., a NIH Training Grant to C.D., and a Korean Government Fellowship to K.B.

REFERENCES

Asada M, Shuler ML (1989) Stimulation of ajmalicine production and excretion from *Catharanthus roseus*: effects of adsorption in situ, elicitors and alginate immobilization. App Microbiol Biotechnol **30**: 475-481

Briggs GE, Haldane JBS (1925) A note on the kinetics of enzyme action. Biochem J **19**: 338-339

Brodelius P, Pederson P (1993) Increasing secondary metabolite production in plant-cell culture by redirecting transport. Tibtech **11**: 30-36

Byun SY, Pederson H, Chin CK (1990) Two phase culture for the enhanced production of benzophenanthridine alkaloids in cell suspensions of *Eschscholtzia californica*. Phytochemistry **29**: 3135-3139

Corry JP, Reed WL, Curtis WR (1993) Enhanced recovery of solavetivone from hairy root cultures of *Hyoscyamus muticus* using integrated product extraction. Biotechnol Bioeng **42**: 503-508

Dische Z (1961) General color reactions. Methods Carbohydr Chem **1**: 479-512

Dixon M, Wedd EC (1979) Enzymes. Longman Group Ltd., London.

Flores HE, Curtis WR (1992) Approaches to understanding and manipulating the biosynthetic potential of plant roots. Proc N Y Acad Sci **655**: 188-209

Funk C, Brodelius PE (1990) Phenylpropanoid metabolism in suspension cultures of *Vanilla planifolia* Andr. II. Effects of precursor feeding and metabolic inhibitors. Plant Physiol **94**: 95-101

Knobloch KH, Berlin J (1980) Influence of medium composition in the formation of secondary compounds in cell suspension cultures of *Catharanthus roseus*. Z Naturforsch **35**: 551-556

Inomata S, Yokoyama M, Seto S, Yanagi M (1991) High-level production of arbutin from hydroquinone in suspension cultures of *Catharanthus roseus* plant cells. Appl Microbiol Biotechnol **36**: 315-319

Michaelis L, Menten ML (1913) Die kinetic der invertinwirkung. Biochem Z **49**: 333-369

Newsholme EA, Start C (1973) Regulation in metabolism. Chap 1, Wiley & Sons, New York, pp 12-56

Panda AK, Bisaria VS, Mishra S (1992) Akaloid production by plant cell cultures of *Holarrhena antidysenterica*. II. Effect of precursor feeding and cultivation in stirred tank bioreactor. Biotechnol Bioeng **39**: 1052-1057

Reddy GR, Signs MW, Flores HE, Curtis WR (1993) Reversed-phase liquid chromatographic isolation of lubimin and solavetivone from *Hyoscyamus muticus* 'hairy' root cultures. J Chromatography **657**: 440-444

Suga T, Hirata T, Hamada S (1988) Biotransformation of 3-oxo-p-methane derivatives by cultured cells of *Nicotiana tabacum*. Phytochemistry **27**: 1041-1044

Threlfall DR, Whitehead IM (1988) Co-ordinated inhibition of squalene synthetase and induction of enzymes of sesquiterpene phytoalexin biosynthesis in cultures of *Nicotiana tabacum*. Phytochemistry **27**: 2567-2580

van der Wielen L, Luyben K (1992) Integrated product formation and recovery in fermentation. Current Opin Biotechnol **3**: 130-138

Vogeli U, Chappell J (1988) Induction of sesquiterpene cyclase and suppression of squalene synthetase activities in plant cell cultures treated with fungal elicitor. Plant Physiol **88**: 1291-1296

Vogeli U, Chappell J (1990) Regulation of a sesquiterpene cyclase in cellulase-treated tobacco cell suspension cultures. Plant Physiol **94**: 1860-1866

(1997년 5월 1일 접수)