รายงานวิจัยฉบับสมบูรณ์

โครงการ การควบคุมเอนไซม์สังเคราะห์ฮอร์โมนลอกคราบ (Moulting hormone) ในเซลล์พืชไข่เน่า (*Vitex glabrata* R.Br.) เพาะเลี้ยง

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คณะผู้วิจัย สังกัด

ภาควิชาเทคโนโลยีชีวภาพ คณะเทคโนโลยี

มหาวิทยาลัยขอนแก่น

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บทคัดย่อ

การผลิตสารเบตา-เอคไดโซนโดยการเพาะเลี้ยงเซลล์แขวนลอยต้นไข่เน่าด้วยการศึกษาปัจจัยต่างๆ ได้แก่ สูตรอาหาร สารควบคุมการเจริญเติบโตและความหนาแน่นของเซลล์เริ่มต้นต่อการเจริญและการผลิตสารเบตา-เอคไดโซน พบว่าเซลล์ที่เพาะเลี้ยงเจริญและผลิตสารเบตา-เอคไดโซนสูงสุดในอาหารสูตร Gamborg's B5 ที่เสริมด้วย BAP 2.0 มิลลิกรัมต่อลิตร และ 2,4-D 1.0 มิลลิกรัมต่อลิตร โดยใช้ความหนาแน่นของเซลล์เริ่มต้น 20 เปอร์เซ็นต์ ให้ผลผลิตเบตา-เอคไดโซนเท่ากับ 1.1 มิลลิกรัมต่อลิตรต่อวัน การเพิ่มผลผลิตของเบตา-เอคไดโซน โดยการศึกษาชนิดของสารต้นตอ (precursor) ต่อการเจริญและการผลิตสารเบตา-เอคไดโซน พบว่าโคเลสเตอรอลที่ความเข้มข้น 100 และ 200 มิลลิกรัมต่อลิตร ยับยั้งการเจริญและมีผลทำให้การผลิตสารเบตา-เอคไดโซนลดลง ในขณะที่สาร 7-ดีโฮโดรโคเลสเตอรอล และเออร์โกสเตอรอล สามารถเพิ่มผลผลิตเบตา-เอคไดโซนได้ โดยเซลล์ที่เพาะเลี้ยงในอาหารที่เติม 7-ดีโฮโดรโคเลสเตอรอล 10 มิลลิกรัมต่อลิตร ผลิตสารเบตา-เอคไดโซน สูงสุดเท่ากับ 1.31 มิลลิกรัมต่อลิตรต่อวัน

เซลล์พืชไข่เน่าที่คัดเลือกได้มีการเจริญและผลิตสารเบตา-เอคไดโซนสูง ใช้เป็นแหล่งในการศึกษาเอนไซม์เอคไดโซนทเวนตีโมโนอ๊อกซิจีเนส (ecdysone 20-monooxygenase) พบว่าโคลนที่คัดเลือกได้สามารถผลิตสารเบตา-เอคไดโซนสูง เท่ากับ 0.047% DW การพัฒนาวิธีการวัดแอคติวิตีของเอคไดโซนทเวนตีโมโนอ๊อกซิจีเนส จากไมโครโซมที่แยกได้จากเซลล์ไข่เน่าเพาะเลี้ยงซึ่งคัดเลือกได้ โดยใช้เครื่องโครมาโตกราฟีของเหลวสมรรถนะสูง (HPLC) พบว่าเอนไซม์ที่แยกได้สามารถเร่งปฏิกิริยาการเปลี่ยนอัลฟา-เอคไดโซนเป็นเบตา-เอคไดโซนได้ โดยปริมาณสารเบตา-เอคไดโซนที่เกิดขึ้นจากการทำงานของเอนไซม์ มีความสัมพันธ์กับระยะเวลาที่ใช้ในการบ่มและปริมาณโปรตีนที่เพิ่มขึ้น การศึกษาคุณสมบัติของเอคไดโซนทเวนตีโมโนอ๊อกซิจิเนส พบว่าอุณหภูมิที่เหมาะสมต่อการเร่งปฏิกิริยาของเอนไซม์ เท่ากับ 32 ^{0}C พีเอช (pH) ที่เหมาะสมต่อการเร่งปฏิกิริยาของเอนไซม์ เท่ากับ 7.2 นอกจากนี้ยังพบว่า NADPH และก๊าซอ๊อกซิเจนจำเป็นต่อการทำงานของเอนไซม์ คาร์บอนมอนน๊อกไซม์ (CO) และสารยั้บยั้งเอนไซม์ในกลุ่มไซโตโครม พี-450 โมโนอ็อกซิจีเนส (cytochrome P450 monooxygenase) ได้แก่ cytochrome c, azadirachtin และ plumbagin ยั้บยั้งการทำงานของเอนไซม์ได้ด้วย จากคุณสมบัติของเอนไซม์ดังกล่าวแสดงให้เห็นว่า เอคไดโซนทเวนตีโมโนอ๊อกซิจีเนส เป็นเอนไซม์ในกลุ่มไซโตโครม พี-450 โมโนอ็อกซิจีเนส

การศึกษาผลของฟีโนบาร์บิทอลซึ่งเป็นสารชักนำการทำงานของเอนไซม์ในกลุ่มไซโตโครม พี-450 โมโนอ็อกซิจีเนส ต่อการเจริญและผลิตสารเบตา-เอคไดโซนพบว่า สารฟีโนบาร์บิทอลเข้มข้น 10 มิลลิกรัมต่อลิตร มีผลทำให้เซลล์ไข่เน่าเจริญ และผลิตสารสารเบตา-เอคไดโซนสูงขึ้น

7.5 เท่า การโคลนยีนที่ควบคุมการสังเคราะห์เอนไซม์เอคไดโซนทเวนตีโมโนอ๊อกซิจีเนส ด้วยเทคนิคพีซีอาร์ (PCR) โดยใช้เอ็มอาร์เอ็นเอ (mRNA) ที่แยกได้จากเซลล์ไข่เน่าเพาะเลี้ยงที่เติมสารฟีโนบาร์บิทอลเป็นต้นแบบในการสังเคราะห์ cDNA ผลการทดลองพบว่า partial cDNA ที่แยกได้ คือ VGP450-1 มีลำดับกรดอะมิโนที่อยู่ในบริเวณอนุรักษ์ (conserved region) ของยีนในกลุ่มไซโตโครม พี-450 โมโนอ็อกซิจีเนส จากการศึกษาความสัมพันธ์ระหว่างการแสดงออกของยีน VGP450-1, แอคติวิตี้ของเอนไซม์เอคไดโซนทเวนตีโมโนอ๊อกซิจีเนส และการผลิตสารเบตา-เอคไดโซนในเซลล์ไข่เน่าที่เติมสารชักนำฟีโนบาร์บิทอล พบว่าการแสดงออกของยีน VGP450-1 มีความสัมพันธ์กับแอคติวิตีของเอนไซม์เอคไดโซนทเวนตีโมโนอ๊อกซิจีเนส และการสะสมสารเบตา-เอคไดโซนที่เพิ่มขึ้น จึงเป็นไปได้ว่าการสังเคราะห์สารเบตา-เอคไดโซนอาจถูกควบคุมด้วยการทำงานของยีน VGP450-1 และยีน VGP450-1 อาจเป็นยีนที่ควบคุมการสังเคราะห์เอนไซม์ ecdysone 20-monooxygenase

ABSTRACT

The effects of the cultivation media, plant growth regulators and inoculum size on the growth and 20-hydroxyecdysone production of suspension cultures of *Vitex glabrata* R.Br. were investigated. The cell growth and maximum 20-hydroxyecdysone production reach the highest when cultured cells in the Gamborg's B5 medium supplemented with 2.0 mg l^{-1} BAP and 1.0 mg l^{-1} 2,4-D. The maximum 20-hydroxyecdysone productivity of about 1.1 mg l^{-1} day^{-1} was observed in the culture with 20% PCV of inoculum size. Enhancement of 20-hydroxyecdysone by precursor feeding on cell growth and 20-hydroxyecdysone production of *V. glabrata* suspension cultures were studied. The addition of cholesterol inhibited growth and decreased the level of 20-hydroxyecdysone. Feeding of 7-dehydrocholesterol and ergosterol increased 20-hydroxyecdysone production. The maximum 20-hydroxyecdysone productivity of about 1.31 mg/l/day was observed in the culture with 10 mg/l 7-dehydrocholesterol added.

Cell lines selection following single cell cloning or cell aggregate cloning was carried out to select cell lines capable of fast growing and high producing level of beta-ecdysone. This maximum level of beta-ecdysone, 0.047%DW could be obtained from the highest selective clones. The selective cell lines was selected for further investigation on ecdysone 20-monooxygenae activity. The microsomal fraction isolated from suspension cells of *V. glabrata* culture was able to catalyse alpha-ecdysone to beta-ecdysone. The HPLC assay was validated with respect to the incubation time and the amount of protein. To characterize the ecdysone 20-monooxygenase, a pH optimum of 7.2 and a temperature optimum of 32 ^{o}C were determined. It was dependent of NADPH and molecular oxygen. The enzymatic reaction was inhibited by carbon monoxide as well as by several cytochrome P450 inhibitors, cytochrome c, azadirachtin and plumbagin. This data indicating that ecdysone 20-monooxygenase is a cytochrome P450 monooxygenase.

Effect of phenobarbital on the growth and 20-hydroxyecdysone production were investigated. Phenobarbital (10 mg/L) increase of both cell growth and 20-hydroxyecdysone production. Using PCR strategies based on the conserved amino acid sequences, P450 cDNA fragments were isolated from phenobarbital-treated *V.*

glabrata suspension-cultured cells. The mRNA was isolated from phenobarbital-treated *V. glabrata* cultured cells, as a template for cDNA synthesis. One partial cDNA clones, VGP450-1 contained the heme-binding domain which is highly conserved among plant cytochrome P450s. Expression of VGP450-1 genes as well as accumulation of ecdysone 20-monooxygenase activity was highly correlated with 20-hydroxyecdysone production in phenobarbital-treated *V. glabrata* cultured cells, suggesting that 20-hydroxyecdysone biosynthesis is regulated at the transcriptional level of the VGP450-1 gene.

EXECUTIVE SUMMARY

The effects of the cultivation media, plant growth regulators and inoculum size on the growth and 20-hydroxyecdysone production of suspension cultures of *Vitex glabrata* R.Br. were investigated. The cell growth and maximum 20-hydroxyecdysone production reach the highest when cells were cultured in the Gamborg's B5 medium supplemented with 2.0 mg l^{-1} BAP and 1.0 mg l^{-1} 2,4-D. The maximum 20-hydroxyecdysone productivity, of about 1.1 mg l^{-1} day^{-1}, was observed in the culture with 20% PCV of inoculum size. These data also show that increment of the inoculum size to 20% PCV could increase 7-folds of productivity.

The effect of ecdysteroid precursors feeding on cell growth and 20-hydroxyecdysone production of *Vitex glabrata* suspension cultures were studied. The addition of cholesterol, there was no appearance increased of 20-hydroxyecdysone contents while partially inhibited growth at higher levels. Feeding of 7-dehydrocholesterol and ergosterol did not affect the cell growth. Both precursors effectively increased production of 20-hydroxyecdysone. The maximum 20-hydroxyecdysone productivity of about 1.31 mg/l/day was observed in the culture with 10 mg/l 7-dehydrocholesterol. This data is the first indication that 7-dehydrocholesterol and ergosterol feeding are an effective precursors for 20-hydroxyecdysone biosynthesis pathway in plant cell suspension culture.

Cell lines selection following single cell cloning or cell aggregate cloning was carried out to select cell lines capable of fast growing and high producing level of beta-ecdysone. Under this method there appeared to be several clones which shown higher growth and beta-ecdysone production, in comparison to the original culture. The level of 0.03-0.047 grams percents of beta-ecdysone could be obtained from the highest selective clones. This maximum level of beta-ecdysone, 0.047% DW was reached within 12 days of cultivation. The selective cell lines was selected for further investigation on ecdysone 20-monooxygenae activity. The preparation of a microsome fraction from *V. glabrata* cultured cells by Mg^{2+} precipitation was developed. Results showed that the microsome preparation isolated from suspension cells of *V. glabrata* culture was able to catalyse alpha-ecdysone to beta-ecdysone. An HPLC assay was developed for the enzyme assay based on the UV detection of the

product beta-ecdysone on a reverse-phase C-18 column. The developed HPLC assay was validated with respect to the incubation time (linear up to 45 min) and the amount of protein added per incubation (linear up to 200 μg of protein).

The activity of ecdysone 20-monooxygenase, an enzyme catalyzing the conversion of ecdysone into 20-hydroxyecdysone, was detected on a microsome preparation from the cell suspension cultures of *V. glabrata*. A pH optimum of 7.2 and a temperature optimum of 32 ^{o}C were determined. It was dependent of NADPH and molecular oxygen. The enzymatic reaction was inhibited by carbon monoxide as well as by several cytochrome P450 inhibitors, especially azadirachtin. The CO inhibition was reversed by light. CO difference spectrum of reduced microsomal fractions gave an absorbance maximum around 450 nm. This data indicated that ecdysone 20-monooxygenase is a cytochrome P450 monooxygenase.

Effects of phenobarbital on the growth and 20-hydroxyecdysone production were investigated. Phenobarbital at doses of 10 mg/L or 20 mg/L stimulate both cell growth and 20-hydroxyecdysone production. Using PCR strategies based on the conserved amino acid sequences, P450 cDNA fragment were isolated from phenobarbital-treated *V. glabrata* suspension-cultured cells. The mRNA was isolated from phenobarbital-treated *V. glabrata* cultured cells and RT-PCR was performed using degenerate primers designed based on the resulted of multi-alignment of six known ecdysone 20-monooxygenase gene from insects. One partial cDNA clones, VGP450-1 showing unique base sequences were obtained. Blast search results show homologies at the levels of nucleotide and amino acid sequences to the others plant cytochrome P450 genes. The deduced amino acid sequences of VGP450-1 contained the heme-binding domain which is highly conserved among plant P450s. Expression of VGP450-1 genes as well as accumulation of ecdysone 20-monooxygenase activity was highly correlated with 20-hydroxyecdysone production in phenobarbital-treated *V. glabrata* cultured cells, suggesting that 20-hydroxyecdysone biosynthesis is regulated at the transcriptional level of the VGP450-1 gene.

I. OPTIMIZATION OF GROWTH AND 20-HYDROXYECDYSONE PRODUCTION OF *Vitex glabrata* R.Br. CELL SUSPENSION CULTURES

Introduction

The ecdysteroid 20-hydroxyecdysone or beta-ecdysone, is one of the moulting hormone which plays a key role in growth, development and reproduction of insects and crustaceans (Butenandt and Karson, 1954). Ecdysteroids and analogues have been used as insecticides (Dhadialla and Tzertzinis, 1998). Moreover, it has been used in the shrimp culture in order to increase productivity (Chaiwatcharakool, 1986). Recent studies on the pharmaceutical properties of 20-hydroxyecdysone, showed it as antimicrobial activity, antidiabetic effect, antioxidative and anti-free radical properties (Lafont and Dinan, 2003). Recently, there are many product containing 20-hydroxyecdysone for oral use can be found on the market. The 20-hydroxyecdysone was isolated from bark of Kai Nao trees or *Vitex glabrata* (Werawattanametin, 1986). Levels of 20-hydroxyecdysone range as high as 2-2.5 % DW from barks of *V. glabrata* was also supported the potential of using this plant as a source of moulting hormone (Thavornnithi, 1990). However, the 20-hydroxyecdysone supply is very limited. It is because of scarcity and slow growth of *V. glabrata* trees. Plant cell culture has been suggested as an attractive alternative that could overcome the limitations of extracting useful metabolites from natural resources (Rao and Ravishan, 2002). Many factors affecting 20-hydroxyecdysone production from *V. glabrata* cultured cells have been investigated, including medium optimization (Thavornnithi, 1990; Prasertsom, 1990), precursors feeding (Prasertsom, 1990). The objectives of this work were to establish cell suspension cultures of *V. glabrata* for the 20-hydroxyecdysone production by manipulating the different medium formulations and type of hormone. The effect of inoculum size on cell growth and 20-hydroxyecdysone production was also studied.

Materials and methods

Plant material and cell cultures

V. glabrata cells initially induced from stem and subcultured for over 12 years at 3-week intervals (Thavornnithi, 1990) were used. Suspension cultures were

established by transferring 40g fresh weight of callus was transferred into 1000 ml Erlenmeyer flasks containing 400 ml of liquid ½ MS medium (Murashige and Skoog, 1962) supplemented with 2.0 mg l^{-1} benzyladenine (BAP) and 1.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). Flasks were placed on a rotary shaker at 120 rpm under continuous light at 2000 lux at 25 ^{o}C. After 3 weeks, cells were filtered through a 1400 μm pore diameter nylon and washed with fresh medium 2 times. Cells were used for the next experiment.

Cell growth determination

Cell growth was determined by measuring the increase in the cell dry weight of the culture. For the determination of cell dry weight, cell suspensions were filtered through Whatman no.1 filter papar on the Buchner funnel under vacuum. The cell were placed on petridish and dried in an oven at 60 ^{o}C for about 2 h to constant weight, then the cell dry weight was determined.

Ecdysteroid extraction and HPLC analysis

Cells were harvested by filtration under suction. A 0.3 g mass of dry cells were extracted with 95% ethanol (180 ml) in a soxhlet apparatus for 6 h. The ethanol extracts were evaporated by rotary evaporator at 60 ^{o}C. The residue was dissolved in methanol (3 ml) and vortexed with hexane (x2, 5 ml). The methanol extracts were evaporated at 60 ^{o}C in hot air oven. The residue was dissolved in distilled water (2 ml). The supernatant was filtered through Sep-pak C_{18} cartridge. Highly polar material was separated from the retained ecdysteroid fraction by elution with distilled water (10 ml). Ecdysteroids were eluted from the cartridge with 20 % methanol-water (10 ml) and the next 80 % methanol-water (10 ml). The supernatant was dried at room temperature in laminar hood. The residue was dissolved in HPLC-grade methanol (1 ml). The supernatant was filtered through a 0.45 μm nylon membrane filter and then dried at room temperature in laminar hood. The residues was redissolved in HPLC-grade methanol (0.1 g cells/100 μl methanol) and analyzed by HPLC. The extracts were analyzed using ODS-3 C18 column (250x4.6mm) The mobile phase consisted of 14% acetonitrile in 2% acetic acid. The flow rate was 1.0 ml min^{-1}. The absorbance was measured at a wavelength of 254 nm.

Results and discussion

Effect of cultivation media

Figure 1 displays the time courses of cell growth and 20-hydroxyecdysone production in suspension cultures in B5 medium and 1/2MS medium, both supplemented with 2.0 mg l^{-1} BAP and 1.0 mg l^{-1} 2,4-D. The highest cell dry weight of 12.1 g l^{-1} was attained in B5 medium on week 3. In the 1/2MS medium, the maximum cell dry weight was found lower than B5 medium by about 20%. HPLC analysis of 20-hydroxyecdysone showed that cells grown on B5 medium produced maximum 20-hydroxyecdysone (0.038% DW) in the third week of cultivation. This value was 24% higher than that using 1/2MS medium. According to the report of Nahalka *et al*. 1996, the plumbagin content in the B5 medium was about 1.8 fold higher than that in the 1/2MS medium. It may be due to the decrease of ammonium ions in the medium. In the present work, B5 medium was effective in promoting both cell growth and 20-hydroxyecdysone production. Hence in this work, B5 medium was chosen as the medium to undergo further investigations.

Effect of plant growth regulators

Effect of plant growth regulators on the cell growth and 20-hydroxyecdysone production of *V. glabrata* cell suspension cultures was also studied (Figure 2). *V. glabrata* cells were cultivated in the B5 medium supplemented with 2.0 mg l^{-1} BAP and 1.0 mg l^{-1} of 2 different auxin (2,4-D and IAA). The cell growth in B5 medium supplemented with 2.0 mg l^{-1} BAP and 1.0 mg l^{-1} IAA reached the maximum growth on week 4 about 7.4 g l^{-1}. Whereas, in B5 medium supplemented with 2.0 mg l^{-1} BAP and 1.0 mg l^{-1} 2,4-D, the maximum cell growth was reached on week 3, 40% higher than that in the medium containing IAA. The maximum 20-hydroxyecdysone content was 0.039% DW in B5 medium supplement with 2.0 mg l^{-1} BAP and 1.0 mg l^{-1} 2,4-D on week 3 of cultivation. This content was 30% greater than that in medium with IAA. Accordingly, B5 medium supplemented with 2.0 mg l^{-1} BAP and 1.0 mg l^{-1} 2,4-D was used in the further experiment.

Effect of inoculum size

The effect of inoculum size on cell growth and 20-hydroxyecdysone production is given in Table 1. The highest cell growth at 20% PCV of inoculum size was obtained at day 4 of cultivation while cells cultured at 10% PCV reached on day 21. An increase in inoculum size greatly shortened the cultivation time. The highest 20-hydroxyecdysone formation of about 0.040% DW was found in the inoculum size of 20% PCV at 4 days of cultivation. The maximum productivity of 20-hydroxyecdysone was 1.10 mg l^{-1} day^{-1} at 20% PCV. This value was 7 folds greater than that using 10% PCV. This indicates that an increase in cell inoculum size is important for increasing 20-hydroxyecdysone production. Akalezi *et al*. (1999) reported that the stimulation of ginseng saponin production by increased inoculum size may be due to cell-to-cell communication and unknown factors released by inoculum size. Moreover, the effect of inoculum size on the cell growth and secondary metabolite production may be related to the enhancement of the activity of the enzymes involved in the metabolic pathway (Contin *et al*., 1998). In the same way, the increase in inoculum size, in order to achieve higher levels of secondary metabolites including taxol for *Taxus chinensis* (Wang *et al*., 1997), cucurbitacin for *Cucurbita andreana* (Halaweish *et al*., 1998) and ginseng saponin for *Panax notoginseng* (Zhang and Zhong, 1997). However, it is still unclear about the detailed mechanism of inoculum size effect on the metabolite biosynthesis.

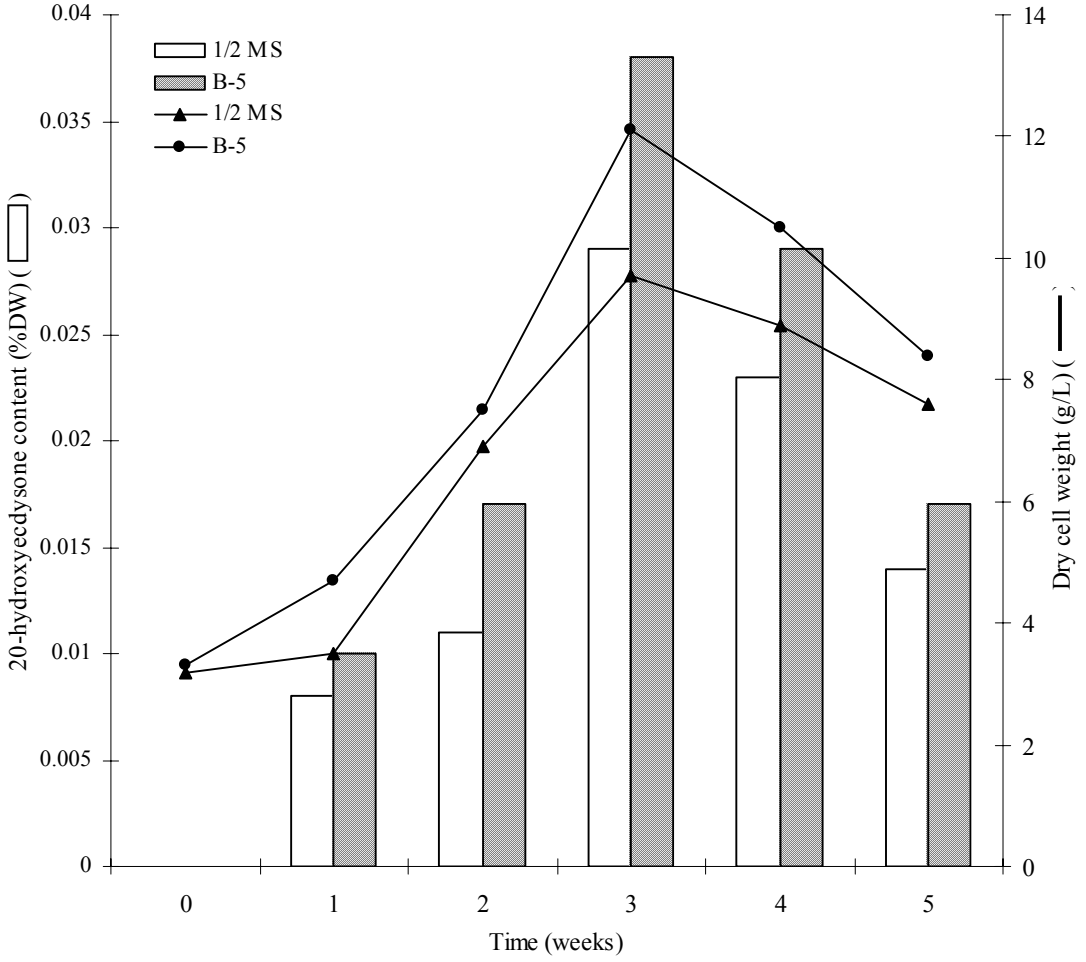

Figure 1 Time course of dry weight and 20-hydroxyecdysone production of *V. glabrata* cell suspension cultures in 2 different media formulation

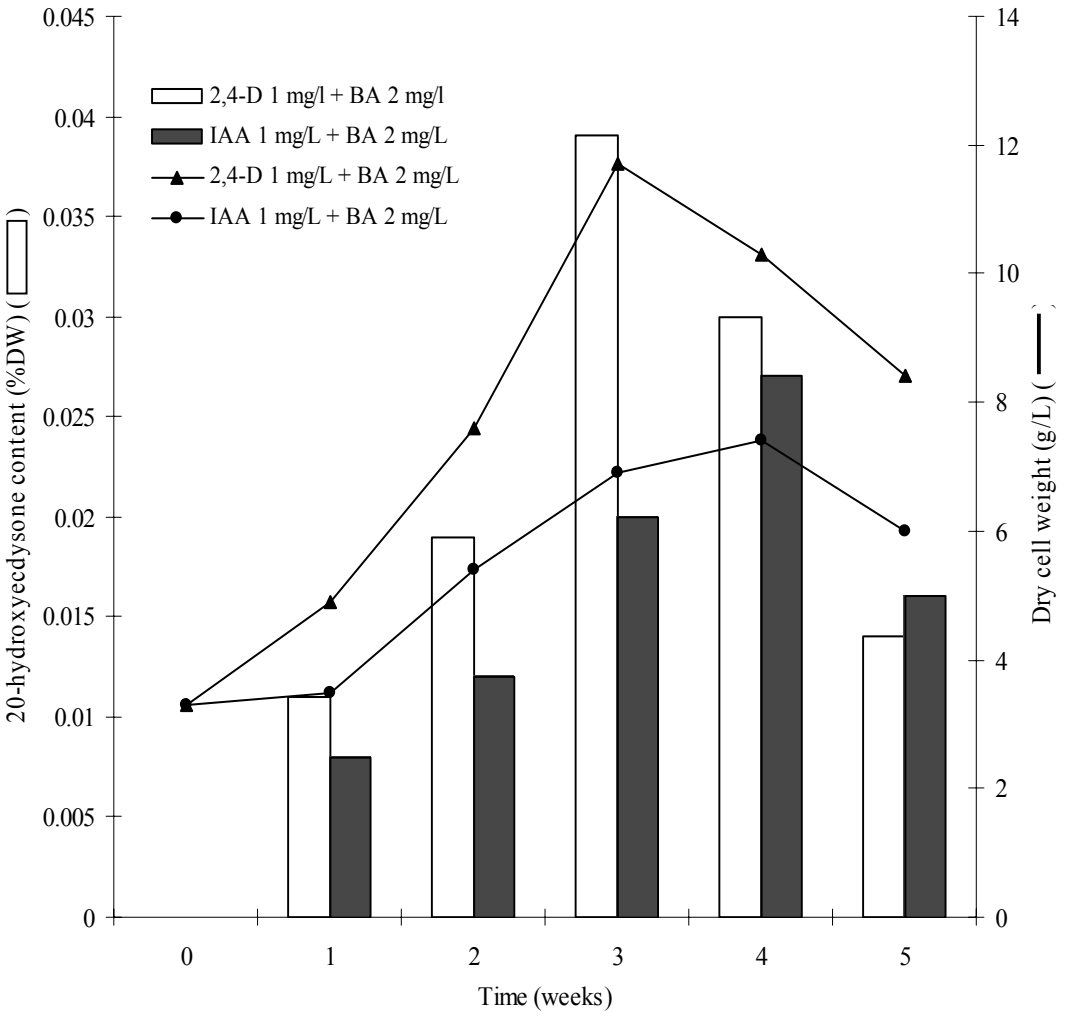

Figure 2 Time course of dry weight and 20-hydroxyecdysone production of *V. glabrata* cell suspension cultures in B5 medium supplement with 2.0 mg/L BAP and 1.0 mg/L of 2 different auxin (2,4-D and IAA)

Table 1 Effect of inoculum size on the biomass and productivity of 20-hydroxyecdysone in suspension culture of *V. glabrata* in B5 medium supplemented with 2.0 mg l^{-1} BAP and 1.0 mg/L 2,4-D.

Inoculum size (% PCV)	Maximum production (% DW)	Maximum cell growth (g l^{-1})	Time of maximum production (day)	Maximum productivity (mg l^{-1} day^{-1})
10	0.033	11.2	21	0.176
20	0.040	11.0	4	1.1

In conclusion, the optimum conditions for the cell growth and 20-hydroxyecdysone production of the cell suspension cultures of *V. glabrata* were in the B5 medium supplemented with 1.0 mg l^{-1} 2,4-D, 2.0 mg l^{-1} BAP and 20% PCV of inoculum size were established.

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II. ENHANCEMENT OF 20-HYDROXYECDYSONE PRODUCTION IN CELL SUSPENSION CULTURES OF *Vitex glabrata* R.Br. BY PRECURSOR FEEDING

Introduction

Vitex glabrata R.Rr. (Verbenaceae) is one of traditional medicinal plant in Thailand, that produce 20-hydroxyecdysone, a phytoecdysteroid, which was first isolated from bark of this plant by Werawattanametin, 1986. The ecdysteroid, 20-hydroxyecdysone is steroid moulting hormone that control growth, moulting and reproduction of arthropods (Butenandt and Karson, 1954). It is found in the most arthropods and also distributed in the plant kingdom. 20-hydroxyecdysone and derivatives were prepared for health improvement, they have been shown to stimulate the synthesis of protein, builds muscle, adaptogenic for HIV patients, antioxidant and tonics properties (Bathori, 2002).

Thavornnithi (1990) isolated very high amounts of 20-hydroxyecdysone about 2.5% DW from barks of *V. glabrata*. However, the 20-hydroxyecdysone production from *V. glabrata* trees is very limited. It is because of scarcity and slow growth of *V. glabrata* trees. Plant cell culture is an alternative route of formation that could overcome the limitation of 20-hydroxyecdysone supply. Studies using plant cell cultures are also of interest for the study of phytoecdysteroid biosynthesis. Many strategies have been followed to increase 20-hydroxyecdysone production from *V. glabrata* cell culture including, medium optimisation (Thavornnithi, 1990, Prasertsom, 1990), precursor feeding (Prasertsom, 1990) and cell line selection (Duanghaklang, 2001). The objective of this study was to investigate the effect of precursors feeding on cell growth and 20-hydroxyecdysone production of *V. glabrata* suspension cultures.

Materials and method

Plant material and culture method

V. glabrata cells, initially induced from stem and subcultured for over 12 years at 3-week intervals, (Thavornnithi, 1990) were used. Suspension culture established with 40 g fresh weight of callus was transferred into 1000 ml Erlenmeyer

flasks containing 400 ml of liquid ½ MS medium (Murashige and Skoog, 1962) supplemented with 2.0 mg l^{-1} benzyladenine (BAP) and 1.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). Flasks were placed on a rotary shaker at 120 rpm under continuous light at 2000 lux at 25 ^{o}C. For 20-hydroxyecdysone production the cells were transferred to the production medium, Gamborg's B5 medium (Gamborg *et al*., 1968) supplemented with 2.0 mg l^{-1} benzyladenine (BAP) and 1.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D).

Addition of precursors

Sterized precursors including 100 and 200 mg/l cholesterol, 1 and 10 mg/l 7-dehydrocholesterol and 1 and 10 mg/l ergosterol, were added to the cell cultures on the day of inoculation. Cells were cultured for several days and harvested for analysis of 20-hydroxyecdysone content and biomass yield. All treatment were performed in duplicate. The cells were harvested after 12, 24, 48, 72, 96 and 120 hrs.

Determination of dry cell weight

For the dry cell weight (DCW) determination, cell suspensions were filtered and washed with distilled water. The fresh cells were dried in an oven at 60 ^{o}C to a constant weight, then the dry cell weight was determined.

Ecdysteroid extraction and analysis

20-hydroxyecdysone was extracted from dried cells as described by Duanghaklang (2001). A 0.3 g mass of dried cells were extracted with 95% ethanol (180 ml) in a soxhlet apparatus for 6 h. The ethanol extracts were evaporated by rotary evaporator at 60 ^{0}C. The residue was dissolved in methanol (3 ml) and vortexed with hexane (x2, 5 ml). The methanol extracts were evaporated at 60 ^{0}C in hot air oven. The residue was dissolved in distilled water (2 ml). The supernatant was filtered though Sep-pak C_{18} cartridge. Highly polar material was separated from the retained ecdysteriod fraction by elution with distilled water (10 ml). Ecdysteroids were eluted from the cartridge with 20% methanol-water (10 ml) and the next 80% methanol-water (10 ml). The supernatant was dried at room temperature in laminar hood. The residue was dissolved in HPLC-grade methanol (1 ml). The supernatant was filtered

through a 0.45 μm nylon membrane filter and then dried at room temperature in laminar hood. The residues was redissolved in HPLC-grade methanol. This solution was subjected to HPLC analysis.

Quantitation of 20-hydroxyecdysone was performed by HPLC using ODS-3 column (250x4.6 mm). The elution was performed by isocratic gradient with 14% acetonitrile in 2% acetic acid. Flow rate was 1.0 ml/min and absorbency at 254 nm was measured.

Result and discussion

Effect of cholesterol feeding on cell growth and 20-hydroxyecdysone production

The effect of cholesterol feeding into Gamborg's B5 medium on cell growth and 20-hydroxyecdysone was shown in Figure 1. The feeding of cholesterol (100 and 200 mg/l) to the cell cultures of *V. glabrata* significantly decreased the growth of the cells over the control culture. It was observed that cholesterol, the early biosynthetic precursor of ecdysteroid pathway, did not increase 20-hydroxyecdysone production. Similarly, Prasertsom (1990) reported that no increase in 20-hydroxyecdysone production in *V. glabrata* cell culture when cholesterol feeding at a concentration 100-200 mg/l.

Effect of 7-dehydrocholesterol feeding on cell growth and 20-hydroxyecdysone production

Figure 2 shows the effect of 7-dehydrocholesterol on cell growth and 20-hydroxyecdysone production on *V. glabrata* suspension cultures. No significant variation in cell growth was observed when the 7-dehydrocholesterol level was varied in the range of 1 and 10 mg/l. The addition of 7-dehydrocholesterol to the cell cultures of *V. glabarata* significantly improved the production of 20-hydroxyecdysone. The maximum amount of 20-hydroxyecdysone was found to be 0.045 %DW on 96 h using 10 mg/l 7-dehydrocholesterol. The increased was about 1.36-fold over the control cultures. This results suggested that improvement of 20-hydroxyecdysone by 7-dehydrocholesterol feeding may be due to its incorporation as a precursors for the biosynthesis of 20-hydroxyecdysone. The earlier reports suggest that 7-dehydrocholesterol is the biosynthetic precursor of ecdysteroid in plant (Ohyama *et*

al., 1999). In addition, Grieneisen *et al*. (1994) reported that radiolabelled 7-dehydrocholesterol was incorporated into 20-hydroxyecdysone in insects.

Effect of ergosterol feeding on cell growth and 20-hydroxyecdysone production

As shown in Figure 3, effect of ergosterol feeding on the cell growth and 20-hydroxyecdysone production of *V. glabrata* cell suspension cultures was also studied. The feeding of ergosterol did not affect the biomass production of the cell culture. The maximum amount of 20-hydroxyecdysone was found to be 0.037 % DW on 96 h using 10 mg/l ergosterol. The increased was about 1.12-fold over the control cultures. This result is the first report that ergosterol feeding can enhance the 20-hydroxyecdysone content.

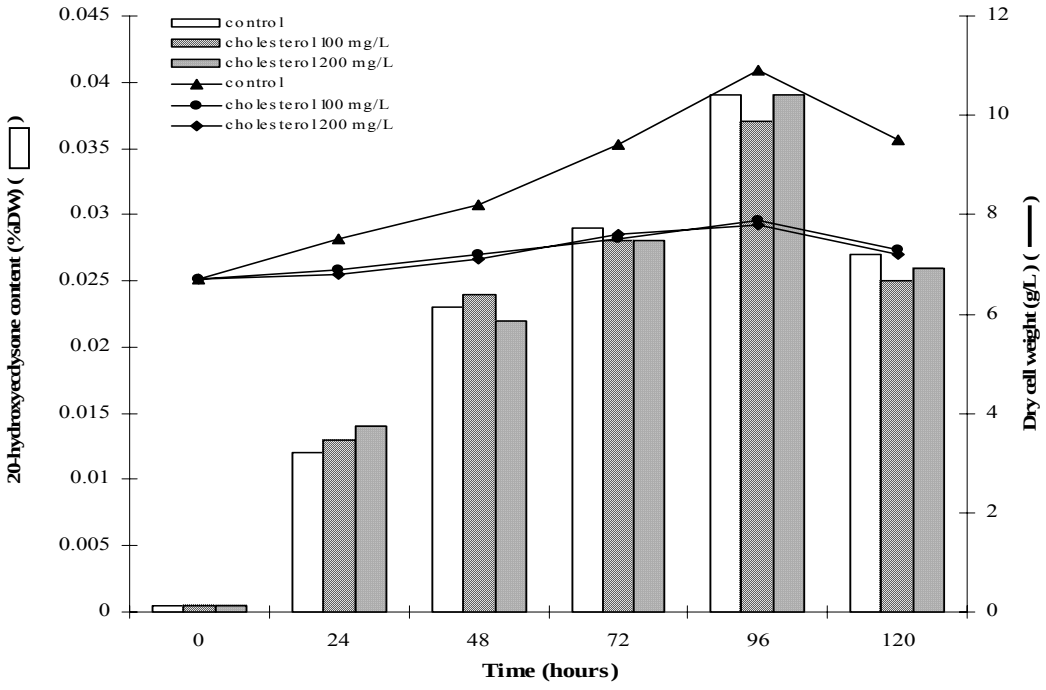

Figure 1 Effect of cholesterol feeding on cell growth and 20-hydroxyecdysone production of *V. glabrata* cell suspension cultures.

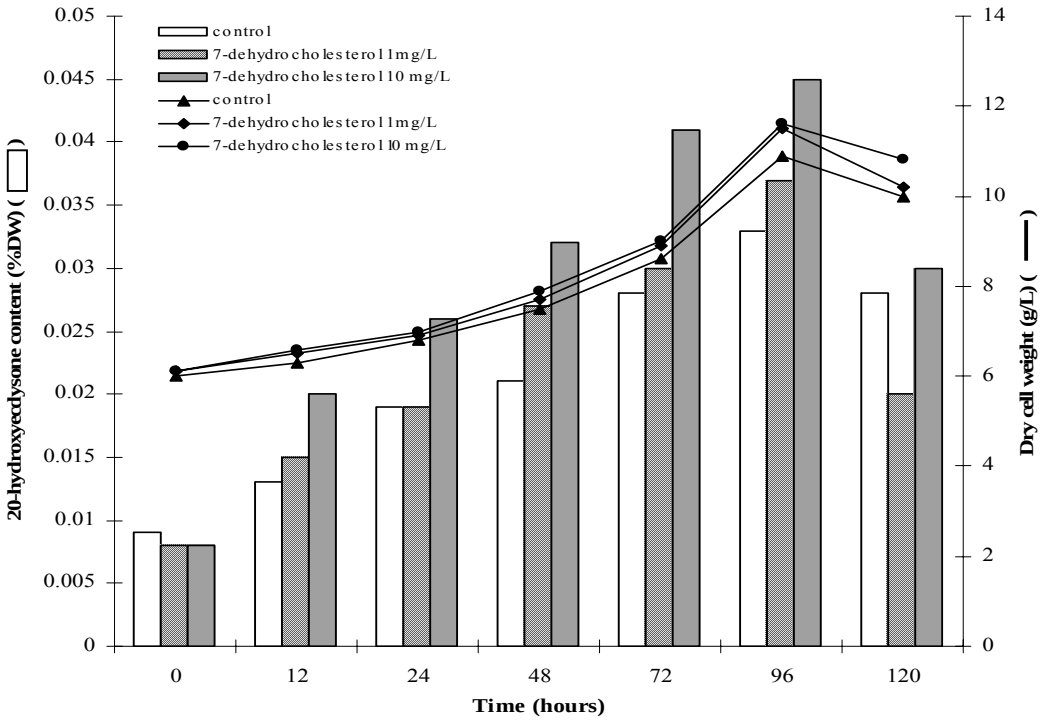

Figure 2 Effect of 7-dehydrocholesterol feeding on cell growth and 20-hydroxyecdysone production of *V. glabrata* cell suspension cultures.

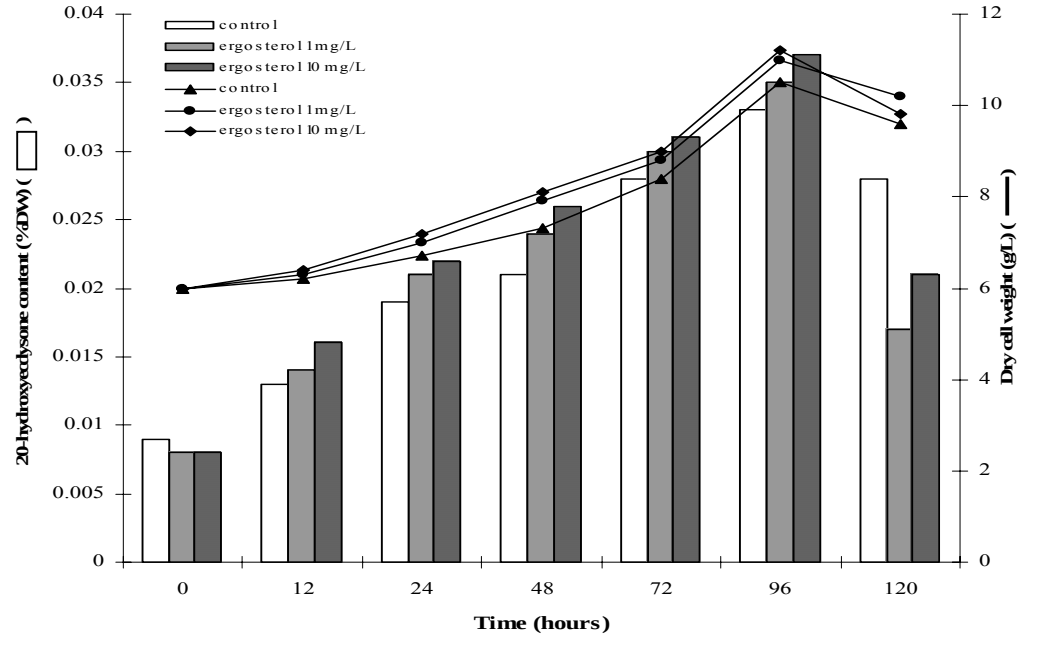

Figure 3 Effect of ergosterol feeding on cell growth and 20-hydroxyecdysone production of *V. glabrata* cell suspension cultures.

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III. SELECTIVE CLONING AND DETECTION OF THE ACTIVITY OF ECDYSONE 20-MONOOXYGENASE IN *Vitex glabrata* R.Br. CELL SUSPENSION CULTURES BY HIGH-PERFORMACE LIQUID CHROMATOGRAPHY

Introduction

The ecdysteroid 20 hydroxyecdysone or β-ecdysone, is one of the moulting hormone which plays a key role in growth, development and reproduction of insects and crustaceans. Ecdysteroids and analogues could be use as insecticides. Moreover, it has been used in the shrimp culture in order to increase productivity (Chaiwatcharakool, 1986). Phytoecdysteroid is known for its pharmacological effect to mammals including against diabetes or asthenia (Lafont and Dinan, 2003). Typically, insects acquire plant sterols like campesterol and convert the plant sterol into 20-hydroxyecdysone. However, some plants accumulate ecdysteroids constitutively in what is thought to be a premature defense response that could serve to induce premature developmental changes and death if ingestion by chewing insects. Accumulation of 20-hydroxyecdysone has been reported for several plants, but is especially high in the bark of *V. glabrata* (Thavornnithi, 1990). The biosynthesis of 20-hydroxyecdysteroid by insects has been established, but little is currently known about this capacity in plants. Plant cell culture has been suggested as an attractive alternative that could overcome the limitations of extracting useful metabolites from natural resources. The optimization of culture medium, precursors feeding, special cell line isolation were used to increase 20-hydroxyecdysone production from *V. glabrata* cultured cells (Duanghaklang, 2001).

Ecdysone 20-monooxygenase (EC 1.14.99.22) is the cytochrome P450-dependent hydroxylase responsible for the conversion of ecdysone to 20-hydroxyecdysone. Grebenok *et al*. (1996) have isolated and characterized ecdysone 20-monooxygenase from spinach, but much remains to be determined. Ecdysone 20-monooxygenase was also detected in intact plant and callus cell culture *of Ajuga reptans* (Alekseeva, 2004) and *Polypodium vulgare* (Canals *et al*., 2005). However, until now there are no reports about the ecdysone 20-monooxygenase in plant cell suspension cultures. The aim of the current study was to select for a high 20-

hydroxyecdysone accumulating tissue culture line of *V. glabrata*, then to use this line as a source of the ecdysone 20-monooxygenase activity in developing an enzyme assay and biochemical characterization.

Materials and Methods

Plant material and culture methods

Vitex glabrata cells initially induced from stem and subcultured for over 12 years at 3-week intervals (Thavornnithi, 1990) were used. Suspension cultures were obtained by transfer of callus cultures to liquid medium. Approximately 40g fresh weight of callus was transferred into 1000 ml Erlenmeyer flasks containing 400 ml of liquid ½ MS medium. Flasks were placed on a rotary shaker at 120 rpm under continuous light at 2000 lux at 25 ^{o}C. After 3 weeks, cells were filtered through a 1400 μm pore diameter nylon and washed with fresh medium 2 times. Cells were used for the next experiment.

Growth measurement

Cell growth was determined by measuring the increase in the cell dry weight of the culture. For the determination of cell dry weight, cell suspensions were filtered through Whatman no.1 filter paper on the Buchner funnel under vacuum. The cell were placed on petridish and dried in an oven at 60 ^{o}C for about 2 h to constant weight, then the cell dry weight was determined.

Ecdysteroid extraction and analysis

Cells were harvested by filtration under suction. A 0.3 g mass of dry cells were extracted with 95% EtOH (180 ml) in a soxhlet apparatus for 6 hours. The EtOH extracts were evaporated by rotary evaporator at 60 ^{o}C. The residue was dissolved in MeOH (3 ml) and vortexed with hexane (x2, 5 ml). The MeOH extracts were evaporated at 60 ^{o}C in hot air oven. The residue was dissolved in distilled water (2 ml). The supernatant was filtered through Sep-pak C_{18} cartridge. Highly polar material was separated from the retained ecdysteroid fraction by elution with distilled water (10 ml). Ecdysteroids were eluted from the cartridge with 20 % MeOH-H_2O (10 ml) and the next 80 % MeOH-H_2O (10 ml). The supernatant was dried at room

temperature in laminar hood. The residue was dissolved in HPLC-grade MeOH (1 ml). The supernatant was filtered through a 0.45 μm nylon membrane filter and then dried at room temperature in laminar hood. The residues was redissolved in HPLC-grade MeOH (0.1 g cells/100 μl MeOH) and analyzed by HPLC. The extracts were analyzed using ODS-3 C18 column (250x4.6mm) The mobile phase consisted of 14% acetonitrile in 2% acetic acid. The flow rate was 1.0 ml/min. The absorbance was measured at a wavelength of 254 nm.

Clonal selection of *V. glabrata* cultured cells

The cell suspension obtained was filtered through a 1400 μm pore diameter nylon net, to eliminate large clumps cells. Cells inoculum (10 ml) was mixed with 90 ml of 1% agarised culture medium and poured into Petridish at a density of $2x10^5$ cell units/Petridish. The dishes were sealed with parafilm and incubated at 25 ^{0}C under continuous fluorescent illumination as above. After 4 weeks of culture, single cell colony (1 mm in diameter) were isolated and transferred to 1/2MS medium for proliferation, subculturing at 3-week intervals. Each cell colony raised from an individual cell unit (single cells or small aggregates) was designated as a separate cell line and selected to reinitiate cell culture. The criteria for cell line selection were cell growth and beta-ecdysone production of the culture lines. Selected callus lines were subcultured at every 3 weeks interval and maintained as callus stocks.

Preparation of microsome

Cells were harvested by suction filtration. All further step were performed at 0-4 ^{o}C. Frozen cells (30g) were ground in the mortar with liquid nitrogen, 0.3 g PVPP and 60 ml buffer [0.1 M Tris-HCl buffer, pH 7.6, 10 mM DTT, 1 mM EDTA, 1 mM PMSF]. The homogenate was filtered through nylon mesh and centrifuged at 10,000 g for 20 min. The supernatant was adjusted to 50 mM $MgCl_2$ and stirred on ice for 20 min. After centrifugation at 30,000 g for 30 min the sediment was resuspended (using glass homogenizer) in 0.1 M K-Pi buffer, pH 7.6 containing 10 mM DTT, 1 mM EDTA, 1 mM PMSF and used for enzyme assays.

Protein determination

Protein concentration were measured by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Assay of crude ecdysone 20-monooxygenase

The standard reaction mixture (total volume 500 µl) contained 55 µM ecdysone dissolved in methanol, microsome suspension in extraction buffer (0.1 M K-Pi buffer, pH 7.6, 10 mM DTT, 1 mM EDTA, 1 mM PMSF). The reaction was initiated by the addition of 1 mM NADPH to the incubation mixture. After incubation at 30 ^{o}C for 60 min the reaction mixture was extracted three times with 500 µl of n-BuOH and the n-BuOH extracts were combined. After evaporation of the solvent, residues were re-dissolved in 100 µl of methanol and analyzed by HPLC. HPLC condition for ecdysone 20-monooxygenase assay was set according to the procedure described in Duanghaklang (2001).

Results and discussion

Cell growth and beta-ecdysone production in *V. glabrata* suspension cultures

Figure 1 shows the profiles of cell growth and beta-ecdysone accumulation of *V. glabrata* cell suspension culture after 12 day of cultivation in shake flask. A lag phase was not apparent from the point of inoculation to the first sampling time at day 2, an exponential growth phase until day 10. The maximum cell growth attained was 16.2 g/l at the late exponential growth phase at day 10 which was also the highest for beta-ecdysone production (0.038 %DW). Beta-ecdysone show apparent positive correlation with the growth curve. In the present study, it was shown that the beta-ecdysone content was growth dependent. Xu *et al*. (1998) reported that the production of jaceosidin in *Rhodiola sachalinensis* was shown to be growth-dependent in culture. Zhao *et al*. (2001) also reported that *Saussurea medusa* cell suspension culture, displayed a relatively growth curve with an almost growth-associated pattern for salidroside accumulation.

Selection of high beta-ecdysone cell lines

Approximately 40 independent cell lines were developed from single-cell or cell aggregate cloning and assessed for their accumulation of 20-hydroxyecdysone. Several clones were identified for their rapid growth and 20-hydroxyecdysone accumulation. Figure 2 shows the time course of two selective fast growing cell lines of *V. glabrata* which were selected from cell cloning. There was no significant difference in cell growth. Considerable variances were observed among quantities of beta-ecdysone produced. In both clones, the maximum beta-ecdysone production which was produced at day 10 was about 0.047 %DW and 0.041 %DW which was significantly higher than that of the original cell lines. The highest beta-ecdysone production of 0.047 % DW was found in the *V. glabrata* (Clone1) cell cultures during the late exponential phase of growth. Selected cell line, Clone 1 was subcultured at 15 day interval and used for further studies. The beta-ecdysone content of selected *V. glabrata* cell lines was 1.5 times higher than that reported by Duanghaklang (2001).

Detection of the activity of the ecdysone 20-monooxygenase in *V. glabrata* cultured cells by HPLC

The nonradioactive assay for ecdysone 20-monooxygenase activity based on the HPLC analysis were developed. Before measuring the activity of enzyme, we studied the enzyme assay conditions and microsome preparation of crude ecdysone 20-monooxygenase from *V. glabrata* cell cultures. We tested the incubation buffer for assay reaction, no detectable product of 20-hydroxyecdysone in 0.1 M Tris-HCl buffer. So, we used 0.1 K-Pi buffer as the incubation buffer. Solubility of 20-hydroxyecdysterone in organic solvents is different, therefore the influence of organic solvent to extract 20-hydroxyecdysone from reaction mixture was also studied. The result showed that 99% of 20-hydroxyecdysone was isolated from reaction mixture when extracted with n-BuOH while only 33% of 20-hydroxyecdysone came out when using ethyl acetate.

Validation of the enzyme assay by the studies of relationship between the incubation time and the amount of beta-ecdysone reaction product formed was determined (Figure 3A). Production of beta-ecdysone was linear up to 45 minutes. Incubation of the reaction mixture assay for 75 min did not increase the amount of

beta-ecdysone. The relationship between the amount of membrane fraction protein added and the amount of beta-ecdysone formed was also determined. The plotted curve obtained (Figure 3B) was linear up to 200 μg of protein. The amount of beta-ecdysone formed was clearly dependent on the amount of microsomal protein added to the assays.

Ecdysone 20-monooxygenase activity is usually measured by a radiochemical method, for example in research of Grebenok *et al*. (1996) and Canals *et al*. (2005). This method is combined with TLC analysis to recover the beta-ecdysone formed in the reaction. For routine experiments, however, such an assay is not only time-consuming but also expensive because of the requirement for the labelled substrate alpha-ecdysone. It was found that this HPLC method which made the use of radioactive substrate unnecessary. Moreover, the HPLC assay will be useful tool for the biochemical characterization of ecdysone 20-monooxygenase from cell suspension cultures of *V. glabrata*. To our knowledge, this is the first report of detection of ecdysone 20-monooxygenase in plant cell suspension cultures.

In conclusion, cell line selection increased 20-hydroxyecdysone production from *V. glabrata* cell culture. A maximum accumulation of 0.047% 20-hydroxyecdysone per gm DW within 12 days of subculturing was observed. The selective cell lines was selected for future investigation on ecdsyone 20-monooxygenase activity. Microsome prepared from cell culture using a Mg^{2+} precipitation method was developed and the microsomes were used to characterize the ecdysone 20-monooxygenase activity. The biosynthesis of 20-hydroxyecdysone from ecdysone was measured using an HPLC separation of product from substrate, and was used to establish several optimal conditions for the assay. Reactions with 50 to 200 μg of microsomal protein were linear up to 45 min when incubations were performed with 0.1 M K-Pi buffer at pH 7.6 and 30 ^{o}C. Further characterization and regulation of this enzyme activity is currently be pursued.

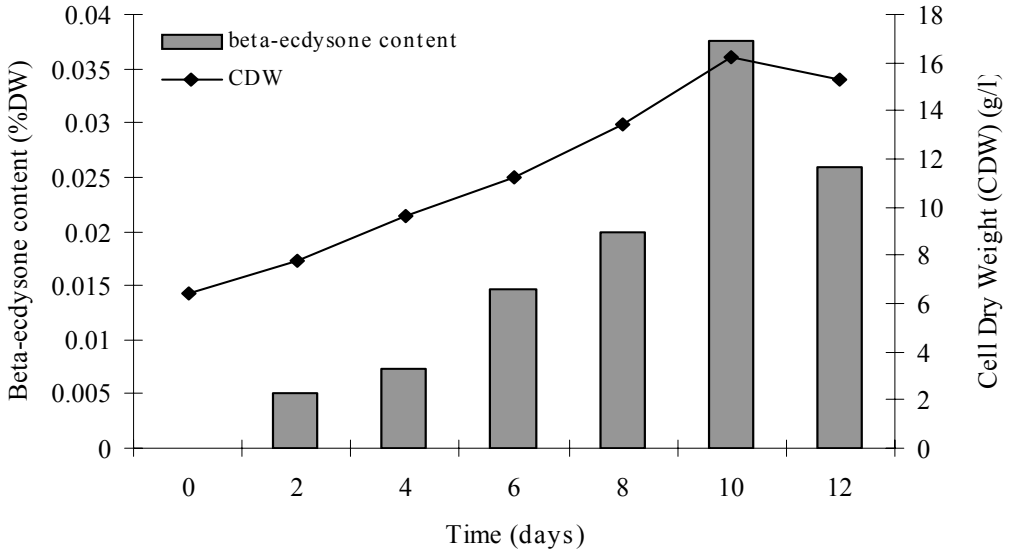

Figure 1 Time course of cell growth and beta-ecdysone production of *V. glabrata* cell suspension culture in B5 medium supplemented with 1.0 mg/L 2,4-D and 2.0 mg/L BAP.

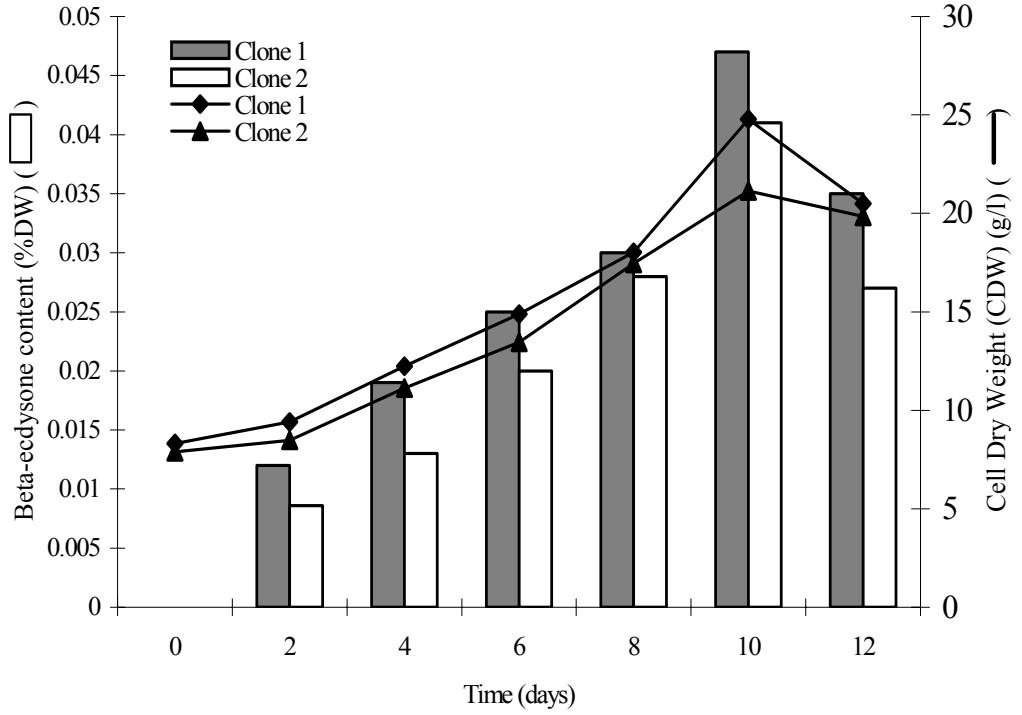

Figure 2 The comparison of beta-ecdysone production and growth of *V. glabrata* two selective cell lines cultured in B5 medium supplemented with 1.0 mg/L 2,4-D and 2.0 mg/L BAP.

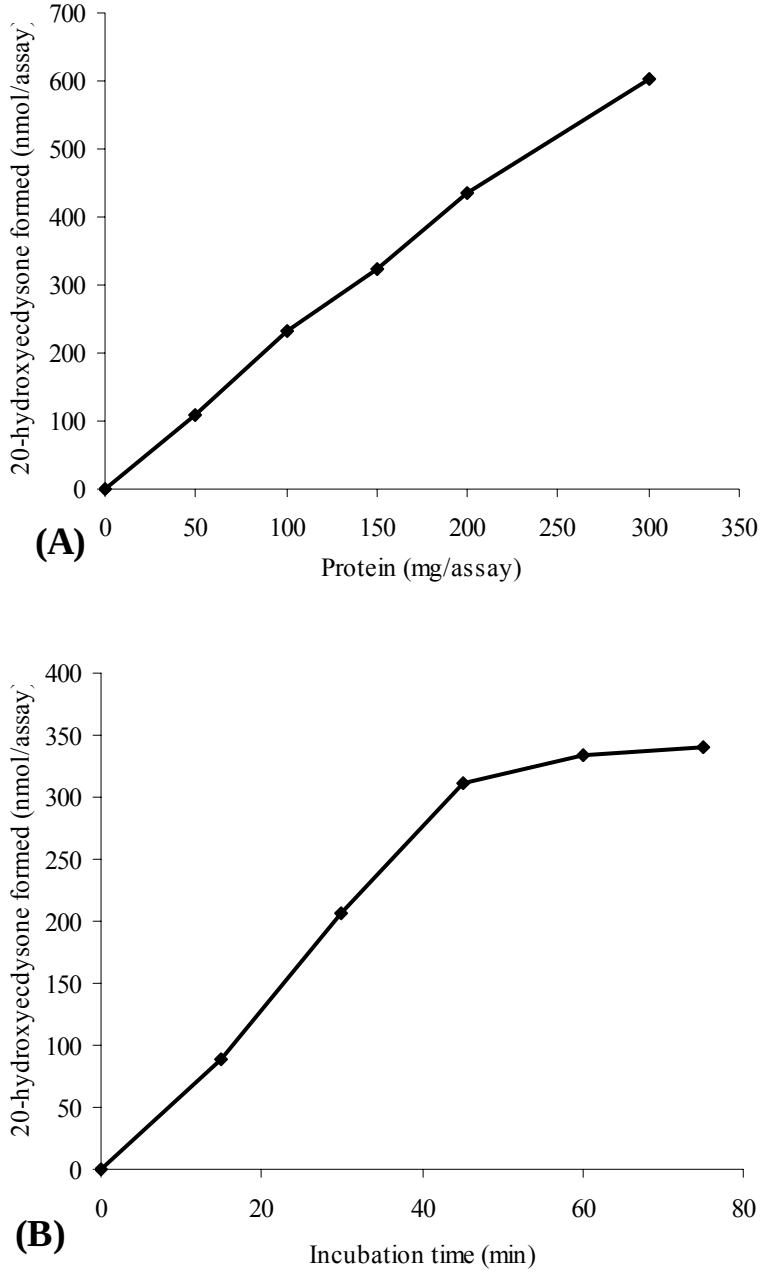

Figure 3 Amount of beta-ecdysone formed during incubation (pmol/incubation volume) in relation to the incubation period in the ecdysone 20-monooxygenase assay(A) and in relation to the amount of membrane protein added in a ecdysone 20-monooxygenase (B).

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IV. ECDYSONE 20-MONOOXYGENASE, A CYTOCHROME P450 MOOXYGENASE ENZYME, FROM CELL CULTURES OF *Vitex glabrata* R.Br.

Introduction

Kai Nao tree or *Vitex glabrata,* belong to family Verbenaceae, is one of traditional medicinal plant in Thailand. The bark and root have reported been used as astringents. The bark has also been claimed to be a anthelmintic and a remedy for gastro-intestinal disorders (Pongboonrod, 1979). 20-hydroxyecdysone was isolated from bark of *V. glabrata* about 2.5 % DW (Thavornnithi, 1990). The ecdysteroid biosynthesis was extensively investigated in insect. The conversion of ecdysone to 20-hydroxyecdysone is the last step in the biosynthesis of the insect molting hormone and is catalyzed by ecdysone 20-monooxygenase (E20M) (Figure 2). This enzyme is a cytochrome P450 monooxygenase located in mitochondria and microsomes of insect homogenates (Nigg *et al*., 1978; Feyereisen and Durst, 1978). The major tissue sources of ecdysone 20-monooxygenase activity are the fat body, midgut, malphighian tubules, integument, and possibly gonads (Smith, 1985). Ecdysone 20-hydroxylation is an essential step in moulting hormone biosynthesis; thus ecdysone 20-monooxygenase plays a crucial role in insect development and reproduction (Smith, 1985). Ecdysone 20-monooxygenase is an important regulatory step in invertebrate ecdysteroids biosynthesis and has been extensively examined in several system, where it has been characterized as a cytochrome P450 (Feyereisen and Durst, 1978).

The biosynthesis of ecdysteriod in plant is not well understood (Adler and Grebenok, 1997). In plant, ecdysone 20-monooxygenase was first isolated and characterized from microsomal fraction of leaves of spinach, *Spinach oleracea* (Grebenok *et al*., 1996). This enzyme is a cytochrome P450 enzyme located in microsome. Recently, Ecdysone 20-monooxygenase was also detected in other plant, for example *Ajuga reptans* (Alekseeva, 2004) and *Polypodium valgarae* (Daniel *et al*., 2005).

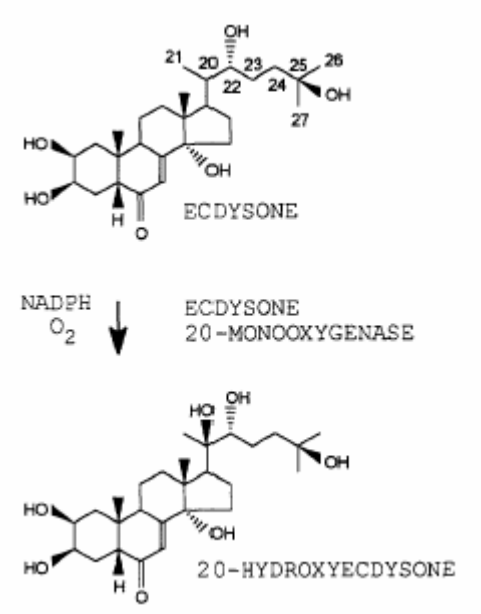

Figure 1 Biosynthetic conversion of ecdysone into 20-hydroxyecdysone (Grebenok *et al*., 1996).

The later enzymatic steps of phytoecdysteroids production are probably similar to those defined for ecdysteroid biosynthesis in arthropods, suggestion that the conversion of ecdysone into 20-hydroxyecdysone in plants may be mediated by a similar cytochrome P450 linked monooxygenase. This study appear to be an ideal model for investigating the biochemistry and enzymology of ecdysteroid production on higher plant. In this research, we report the isolation and partial characterization of ecdysone 20-monooxygenase from *V. glabrata* cell cultures.

Materials and Methods

Plant material and cell culture methods

Vitex glabrata cells initially induced from stem and subcultured for over 12 years at 3-week intervals (Thavornnithi, 1990) were used. Suspension cultures were obtained by transfer of callus cultures to liquid medium. Approximately 40g fresh weight of callus was transferred into 1000 ml Erlenmeyer flasks containing 400 ml of liquid 1/2MS medium. Flasks were placed on a rotary shaker at 120 rpm under continuous light at 2000 lux at 25 ^{o}C. After 3 weeks, cells were filtered through a 1400 μm pore

diameter nylon and washed with fresh medium 2 times. Cells were used for the next experiment.

Microsome preparation

Cells were harvested by suction filtration. All further step were performed at 0-4 ^{o}C. Frozen cells (30g) were ground in the mortar with liquid nitrogen, 0.3 g PVPP and 60 ml buffer [0.1 M Tris-HCl buffer, pH 7.6, 10 mM DTT, 1 mM EDTA, 1 mM PMSF]. The homogenate was filtered through nylon mesh and centrifuged at 10,000 g for 20 min. The supernatant was adjusted to 50 mM $MgCl_2$ and stirred on ice for 20 min. After centrifugation at 30,000 g for 30 min, the sediment was resuspended (using glass homogenizer) in 0.1 M K-Pi buffer, pH 7.6 containing 10 mM DTT, 1 mM EDTA, 1 mM PMSF and used for enzyme assays.

Protein determination

Protein concentration were measured by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Assay of crude ecdysone 20-monooxygenase

The standard reaction mixture (total volume 500 μl) contained 55 μM ecdysone dissolved in methanol, microsome suspension in extraction buffer (0.1 M K-Pi buffer, pH 7.6, 10 mM DTT, 1 mM EDTA, 1 mM PMSF). The reaction was initiated by the addition of 1 mM NADPH to the incubation mixture. After incubation at 30 ^{o}C for 60 min the reaction mixture was extracted three times with 500 μl of n-BuOH and the n-BuOH extracts were combined. After evaporation of the solvent, the residues were re-dissolved in 100 μl of methanol and analyzed by HPLC.

HPLC analysis

The amount of 20-hydroxyecdysone was determined by reversed phase HPLC using ODS-3 C18 column (250x4.6mm, GL Science, Japan) in an oven at 40 ^{o}C. The mobile phase consisted of 14% acetonitrile in 2% acetic acid. The flow rate was 1.0 ml/min, monitoring the absorption at 254 nm.

Identification of the reaction product

The identification of the product was carried out on the basis of UV obtained using a Waters 2695 HPLC-photodiode array system (Waters, USA). The HPLC condition was as follows: solvent, 13% acetonitrile in 2% acetic acid ; at a flow rate of 1.0 ml/min, monitoring the absorption at 254 nm oven temperature, 40^{o}C.

Inhibition experiments

The cytochrome inhibitors, plumbagin, azadirachtin and cytochrome c were tested. The concentration of inhibitors were adjusted to 0.2 mM in the reaction solution. The inhibition experiments were carried out using the standard assay described above.

Carbon monoxide difference spectra of the microsome

Difference spectra, in the 400-500 nm range, were obtained for the microsome preparation by the method of Omura and Sato (1964). Microsome protein was suspended in the extraction buffer, and both sample and blank cuvette were reduced by the addition of sodium dithionite and base line absorbance was recorded. CO was then bubbled through the sample cuvette for 1 min and the difference spectrum recorded, after which air was vigorously bubbled through the sample and the spectrum recorded.

Results and discussion

Characterization of ecdysone 20-monooxygenase

The ecdysone 20-monooxygenase from cell cultures of *V. glabrata* exhibited maximal activity in 0.1 M K-Pi buffer, pH 7.2 and an incubation temperature of 32 ^{o}C (Figure 2 and 3). The ecdysone 20-monooxygenase from larvae of *Drosophila melanogaster*, was found to be most active in a 0.1 M K-Pi buffer, pH 7.5, and exhibited a temperature optimum at 35 ^{o}C (Mitchell and Smith, 1986). The enzymatic properties of *Manduca sexta* ecdysone 20-monooxygenase are that it is most active in a 0.05 M K-Pi buffer and exhibited pH and temperature optimum at 7.5 and 30 ^{o}C (Smith *et al*., 1979). The ecdysone 20-monooxygenase in crab *Gecarcinus lateralis*

showed the maximal activity at 30 ^{o}C and pH 8.0 in sodium phosphate buffer (Soumoff and Skinner, 1988).

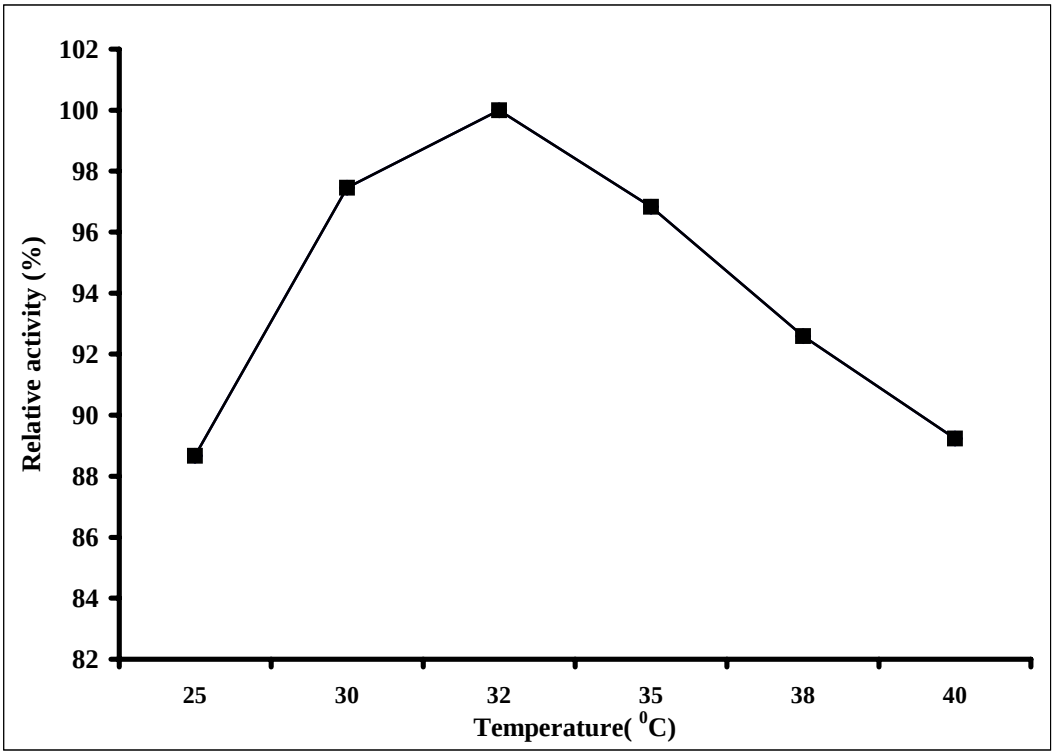

Figure 2 Optimal temperature for the activity of *V. glabrata* ecdysone 20-monooxygenase.

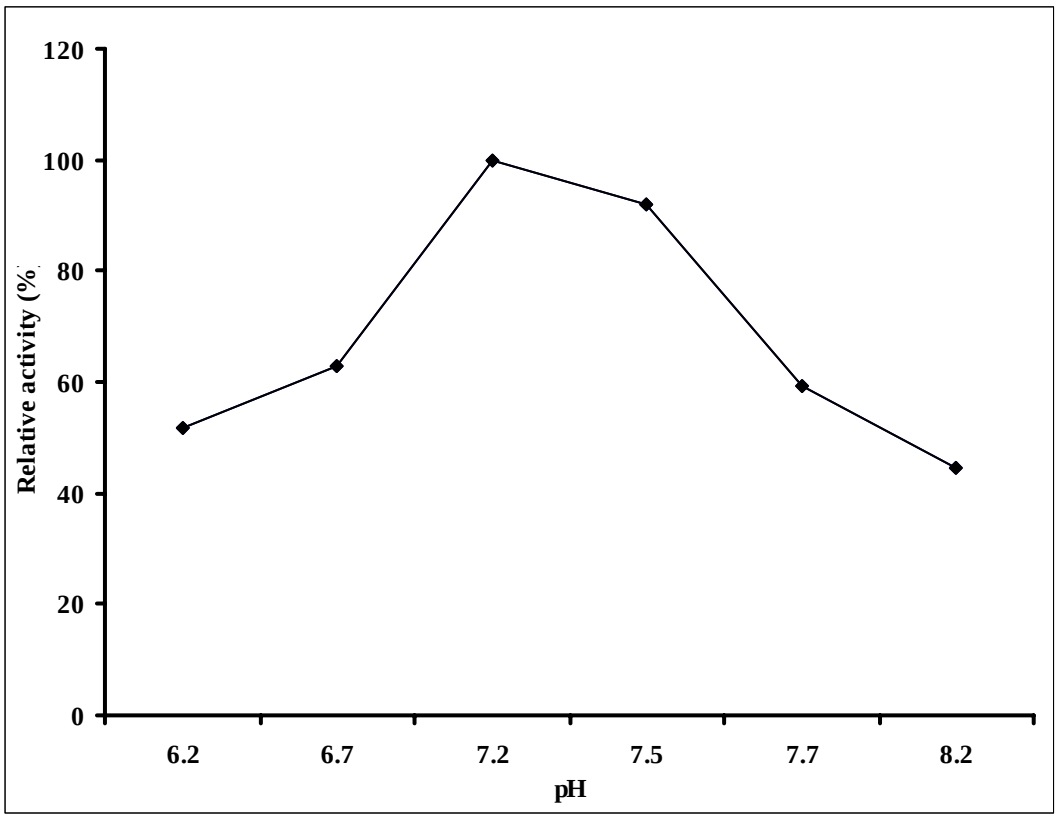

Figure 3 Optimal pH range and the activity of *V. glabrata* ecdysone 20-monooxygenase.

Involvement of cytochrome P450 in ecdysone hydroxylation

In order to investigate the properties of ecdysone 20-monooxygenase, we first examined the cofactor requirement of the hydroxylase by using the microsome fraction. NADPH, NADH and NADP+ were used as potential cofactors for monooxygenase. NADPH was essential for enzymatic activity (Table 1). $NADP^+$ is a competitive inhibitor of NADPH-cytochrome P450 reductase, which is involved in electron transfer in the hydroxylation reaction.When NADPH was substituted with NADH and $NADP^+$, only 44.88 and 38.25% of the activity were detected, respectively.

The effect of molecular oxygen on ecdysone 20-monooxygenase was examined (Table 2). Replacement of O2 in the reaction mixture by N2 prior to the incubation led to a significance decrease in the activity. An absolute requirement for

oxygen was demonstrated by the removal of oxygen from the assay system by addition of the glucose oxidase/catalase system (Kochs and Grisebach, 1987). This inhibition was not observed when glucose oxidase was denatured by heating. This results suggest that the hydroxylation is catalysed by cytochrome P450.

The requirement for NADPH and O2 of the microsomal fraction suggested the participation of a cytochrome P450 in the ecdysone hydroxylation reaction. To confirm this hypothesis, the effects of carbon monoxide (Omura and Sato, 1964) and several cytochrome P450 inhibitors on enzyme activity were examined. A typical characteristic of cytochrome P450 enzymes is their inhibition by carbon monoxide which is tightly bound to the heme moiety of cytochrome P450 instead of O_2. Carbon monoxide, a well-known inhibitor of cytochrome P450 (Ortiz de Montellano and Correia, 1995), strongly inhibited the hydroxylation in dark. Upon illumination with light of the wavelength 450 nm this bond is destabilized and the inhibition is at least reversed (West, 1980). To test this for ecdysone 20-monooxygenase from *V. glabrata*, assays were performed in an atmosphere containing $CO:O_2$ (9:1) in the dark and with illumination with 450-nm light. In the dark, only 18.6% of control ecdysone 20-monooxygenase activity (air) was measured, whereas an activity of 95.3% was observed in the blue-light-illuminated assays (Table 3). Cytochorme c, which competitively remove electrons from the NADPH-cytochrome c reductase complex (Ortiz de Montellano and Correia, 1995), was the strongest inhibitor tested. Other inhibitors, azadirachtin and plumbagin also inhibited enzyme activity. Azadirachtin has been previously shown to be effectively inhibitors of *Manduca sexta* ecdysone 20-monooxygenase (Smith and Mitchell, 1988; Mitchell *et al*., 1997).

The presence of CO-binding pigment in the microsomal fraction from *V. glabrata* cell suspension cultures was confirmed by the CO-difference spectrum (Figure 4). One absorbance peak was seen at 450 nm.

Table 1 Cofactor requirement of ecdysone 20-monooxygenase in *V. glabrata* cell suspension cultures

Cofactors	Relative activity (%)
None	0
2.0 mM NADPH	100
2.0 mM NADH	48.88
2.0 mM $NADP^+$	38.25

Table 2 Oxygen dependence of ecdysone 20-monooxygenase activity

Conditions	Relative activity (%)
Control (Air bubbled)	100
N_2	10.1
Air + glucose + glucose oxidase + catalase	11.3
Air + glucose + boiled glucose oxidase + catalase	89.5

Table 3 Effect of cytochrome P450 inhibitors on ecdysone 20-monooxygenase activity

Inhibitor	Relative activity (%)
Control(Air bubbled)	100
$CO:O_2$ (9:1), dark	18.6
$CO:O_2$ (9:1), light	95.3
0.2 mM azadirachtin	8.1
0.2 mM cytochrome c	7.3
0.2 mM plumbagin	18.9

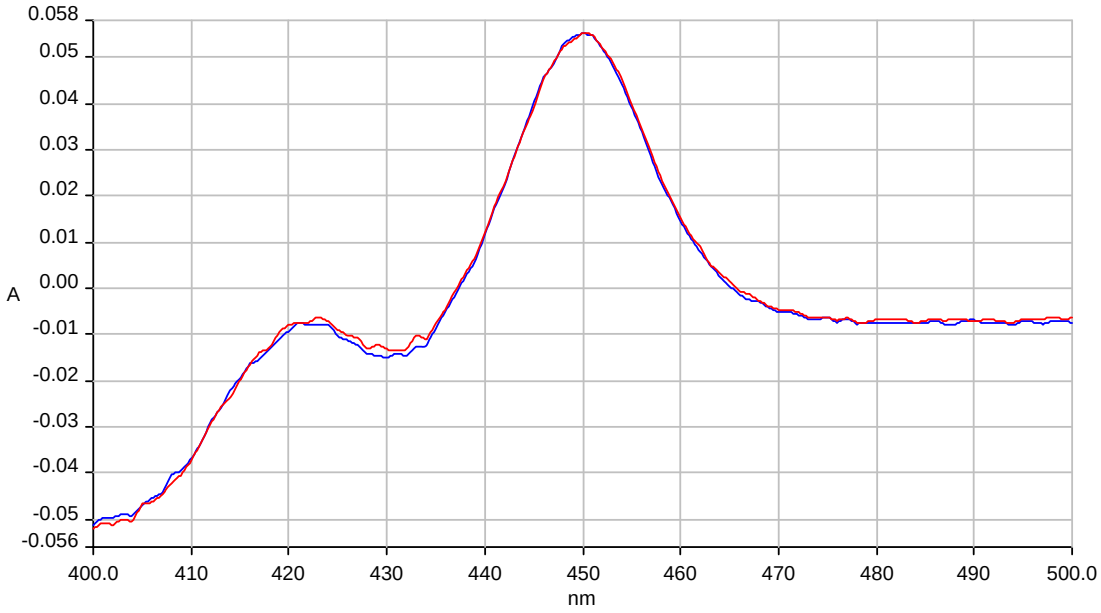

Figure 4 Carbon monoxide difference spectrum of sodium dithionite-reduced soluble cytochrome P450 protein from *V. glabrata* suspension culture.

Growth and ecdysone 20-monooxygenase activity of *V. glabrata* cell cultures

Growth, measured as accumulation of fresh weight, as well as ecdysone 20-monooxygenase activity in *V. glabrata* cell cultures, are shown in Figure 5. Fresh weight increased until day 10 of the culture period. The ecdysone 20-monooxygenase activity continuously increased until the sixth day of cultivation and reached a maximum level of 46.6% of the cell fresh weight. Maximum ecdysone 20-ecdysone activity in *V. glabrata* cell cultures occurred around day 6 at the exponential of growth. At day 10, cell reached the maximum growth and the enzyme activity decreased.

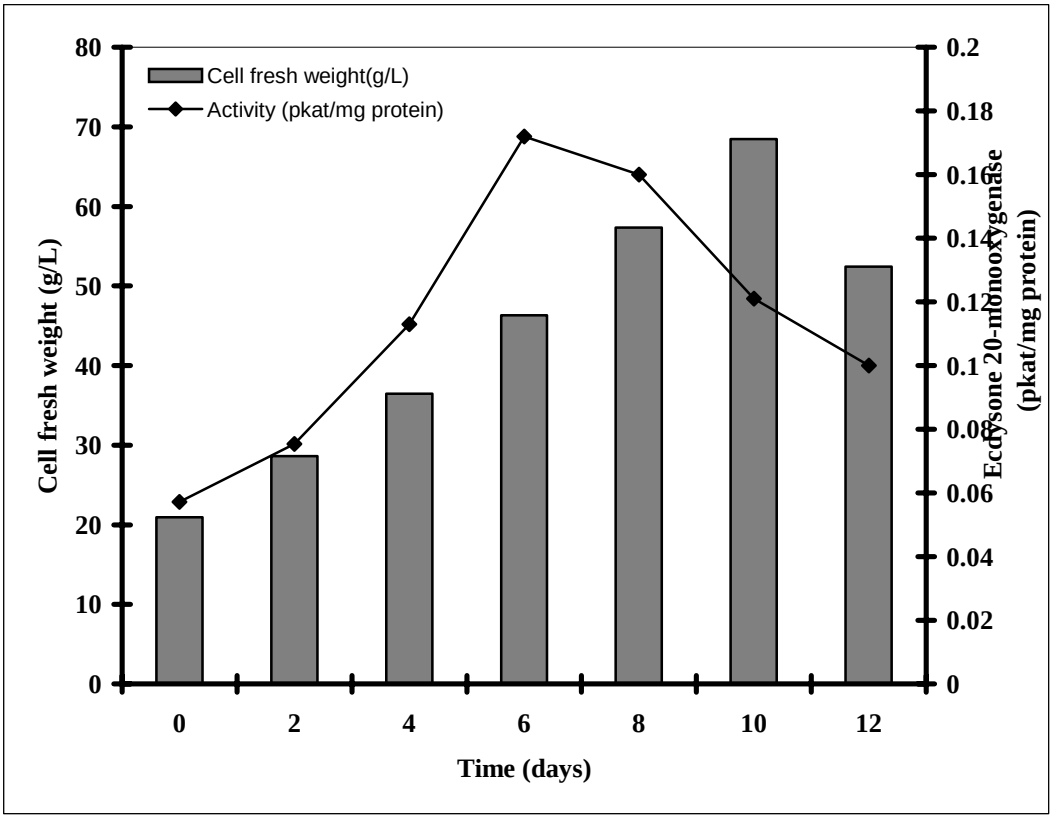

Figure 5 Time courses of cell growth and ecdysone 20-monooxygenase activity in *V. glabrata* cell suspension cultures in Gamborg's B5 medium supplemented with 1.0 mg/L 2,4-D and 2.0 mg/L BAP.

In conclusion, cholesterol feeding did not result in any increase in 20-hydroxyecdysone contents, while inhibiting cell growth in *V. glabrata* cell cultures. 7-dehydrocholesterol and ergosterol feeding led to increase 20-hydroxyecdysone production without affecting cell growth in *V. glabrata* cell cultures. This results indicated the possibility of 7-dehydrocholesterol and ergosterol as the natural precursor for ecdysteroids biosynthetic pathway in plant cells. However, there is a need to study further employing the radiolabeled precursors to show the effect.

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V. MOLECULAR CLONING OF ECDYSONE 20-MONOOXYGENASE FROM *Vitex glabrata* R.Br. CELL CULTURES

Introduction

Insect moulting hormones, ecdysteroids, are reported in both arthropods and higher plants. The production of ecdysteroid such as 20-hydroxyecdysone by arthropods is required for the control of growth, development and reproduction. Phytoecdysteroids are a class of chemicals that synthesize for defense against phytophagous insects. These compounds are exact replicas of ecdysteroids, hormones used by the arthropods(insect) and crustacean families in the molting process known as ecdysis. Insects that ingest phytoecdysteroids and have not adapted to this defense are subjected to serious adverse effects, including reduced weight, molting disruption, and mortarity (Dinan, 2001). The most common phytoecdysteroid found in plants is 20-hydroxyecdysone. The biosynthetic pathway of ecdysteroids in plants is not well understood.

The biosynthesis and metabolism of insect steroid hormone, 20-hydroxyecdysone, requires the action of a number of cytochrome P450. To date, four different enzymes involved in the biosynthetic pathway from dietary cholesterol to 20-hydroxyecdysone have been characterized as cytochrome P450 (Figure 1). The terminal step in the biosynthesis of 20-hydroxyecdysone , which is considered to have a dominant role in molting and development, is catalysed by ecdysone 20-monooxygenase representing the best characterized insect cytochrome P450 to date.

Plant P450s belong to the cytochrome group that are membrane-bound enzymes, usually found in plant endoplasmic reticulum. This gene family is diverse in structure and function, which enables these enzymes to participate in numerous biosynthetic and degradative pathways. Purification of functional P450 enzymes has proven to be difficult due to their low abundance and lability (Chapple, 1998). However, in the last decade, molecular cloning techniques have been successfully used to isolate a large number of P450 genes from many plant species. Recently, sequencing of the complete *Arabidopsis* genome identified a total of 224 cytochrome P450 genes. However, the function of most of these genes is still unknown.

In this chapter, The effect of cytochrome P450 inducer phenobarbital was examined on growth and 20-hydroxyecdysone production in *V. glabrata* cell culture. We have also employed PCR-based strategies to isolate ecdysone 20-monooxygenase genes from *V. glabrata* cell culture. The expression of candidate P450 cDNA clone which isolated from *V. glabrata* cell suspension culture was also studied.

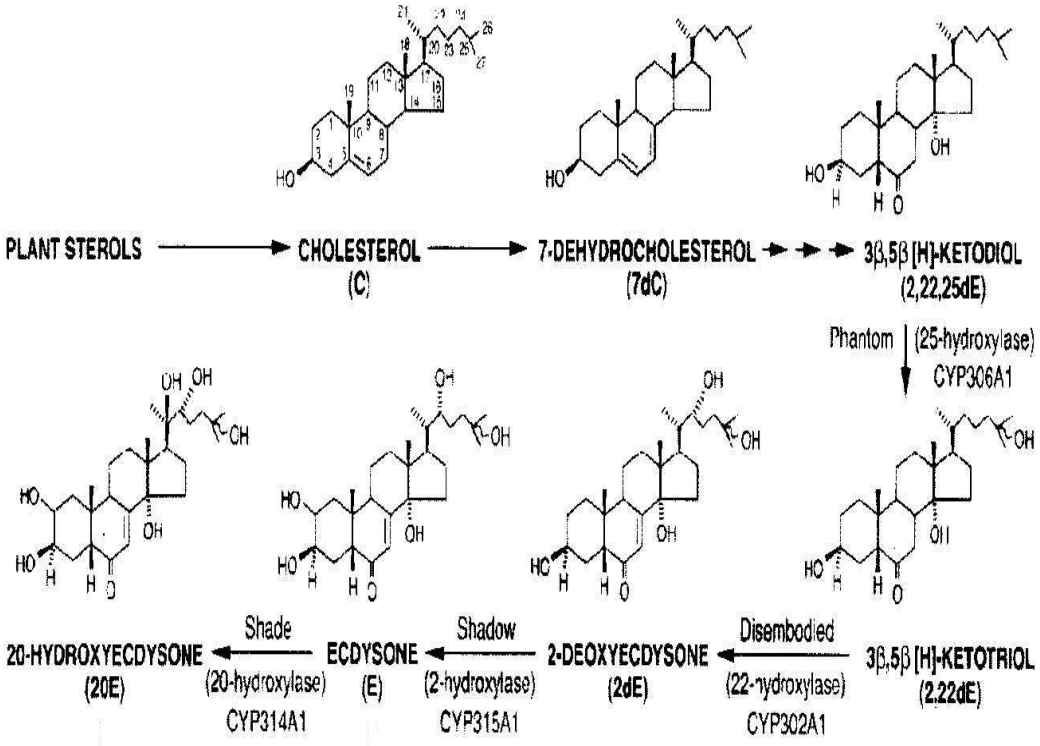

Figure 1 Scheme of 20-hydroxyecdysone biosynthesis. Four cytochrome P450 enzymes have been identified as catalyzing the last four hydroxylation reaction in ecdysteroid biosynthesis (Warren *et al*., 2002)

Materials and methods

Chemicals

Standard laboratory reagents were purchased from Becton Dickinson Microbiology Systems (Sparks, MD), FisherBiotech (Fair Lawn, NJ), Sigma Chemical Company (St. Louis, MO), and Aldrich Chemical Co. (Milwaukee, WI). Ecdysone and 20-hydroxyecdysone was obtained from Severnaya Biochimicheskaya Kompaniya (Russia).

Plant materials, culture methods and induction treatment

Callus cultures of *V. glabrata* were maintained in MS medium (Murashige and Skoog, 1954) supplemented with 3% sucrose, 1.0 mg/L 2,4-Dichlorophenoxyacetic acid and 2.0 mg/L Benzylaminopurine. The cells were subcultured at 25 ^{o}C every week interval. Suspension cultures are initiated by inoculating the callus cells into liquid media of the same composition but without agar. Suspension-cultured cells were grown in 500 ml Erlenmeyer flasks containing 150 ml medium on a rotary shaker at 120 rpm at 25 ^{o}C in the dark. For induction experiments, Phenobarbital was dissolved in water and aseptically added to the cultures through membrane filters at a final concentration of 10 mg/L at the day of inoculation. The cells were collected by vacuum fitration at the defined times, immediately frozen in liquid nitrogen, and stored at –80 ^{o}C. until use.

Ecdysteroids extraction and analysis by HPLC

Cells were harvested by vacuum filtration. A 0.3 g mass of dry cells were extracted with 95% EtOH (180 ml) in a soxhlet apparatus for 6 hours. The EtOH extracts were evaporated by rotary evaporator at 60 ^{o}C. The residue was dissolved in MeOH (3 ml) and vortexed with hexane (x2, 5 ml). The MeOH extracts were evaporated at 60 ^{o}C in hot air oven. The residue was dissolved in distilled water (2 ml). The supernatant was filtered through Sep-pak C_{18} cartridge. Highly polar material was separated from the retained ecdysteroid fraction by elution with distilled water (10 ml). Ecdysteroids were eluted from the cartridge with 20 % MeOH-H_2O (10 ml) and the next 80 % MeOH-H_2O (10 ml). The supernatant was dried at room temperature in laminar hood. The residue was dissolved in HPLC-grade MeOH (1

ml). The supernatant was filtered through a 0.45 μm nylon membrane filter and then dried at room temperature in laminar hood. The residues was redissolved in HPLC-grade MeOH (0.1 g cells/100 μl MeOH) and analyzed by HPLC. The extracts were analyzed using ODS-3 C18 column (250x4.6mm) The mobile phase consisted of 14% acetonitrile in 2% acetic acid. The flow rate was 1.0 ml/min. The absorbance was measured at a wavelength of 254 nm.

Preparation of microsome

Cells were harvested by vacuum filtration. All further step were performed at 0-4 ^{o}C. Frozen cells (30g) were ground in the mortar with liquid nitrogen, 0.3 g PVPP and 60 ml buffer [0.1 M Tris-HCl buffer, pH 7.6, 10 mM DTT, 1 mM EDTA, 1 mM PMSF]. The homogenate was filtered through nylon mesh and centrifuged at 10,000 g for 20 min. The supernatant was adjusted to 50 mM $MgCl_2$ and stirred on ice for 20 min. After centrifugation at 30,000 g for 30 min the sediment was resuspended (using glass homogenizer) in 0.1 M K-Pi buffer buffer, pH 7.6 containing 10 mM DTT, 1 mM EDTA, 1 mM PMSF and used for enzyme assays.

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Assay of crude ecdysone 20-monooxygenase

The standard reaction mixture (total volume 500 μl) contained 55 μM ecdysone dissolved in methanol, microsome suspension in extraction buffer (0.1 M K-Pi buffer, pH 7.6, 10 mM DTT, 1 mM EDTA, 1 mM PMSF). The reaction was initiated by the addition of 1 mM NADPH to the incubation mixture. After incubation at 30 ^{o}C for 60 min, the reaction mixture was extracted three times with 500 μl of n-BuOH and the n-BuOH extracts were combined. After evaporation of the solvent, the residues were re-dissolved in 100 μl of methanol and analyzed by HPLC.

Primer design and isolation of P450 cDNA fragments by PCR cloning strategy

Degenerate oligonucleotide primers used in this study were synthesized by Integrated DNA Technologies, Inc (Caralville, IA). To amplify the P450 cDNA fragment of *V. glabrata*, a primer set (shown in Table 1) was designed from conserved regions identified after multi-alignment of CYP314A1 from several insects*: Drosophila melanogaster*, *Anopheles gambiae*, *Aedes aegypti*, *Manduca sexta*, *Bombyx mori* and *Apis mellifera*.

The cDNA synthesis was performed with 500 ng of mRNA using SMART^{TM} RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's manual. The first strand cDNA was synthesized in a 10 μl reaction mixture at 42 ^{o}C for 1.5 h. After cDNA synthesis, the reaction mixture was diluted to a 260 μl final volume by adding 250 μl of Tricine-EDTA buffer.

PCR was carried out with 2.5 μl of the synthesized cDNA in 50 μl reaction buffer containing 0.2 μM each type-specific primer and 2.5 units of Taq DNA polymerase (Promega, Madison, WI, USA). The PCR amplication products were purified by using QIAquick PCR purification kit (Qiagen) and then the purified PCR product was cloned into pGEM®-T easy vector (Promga, Madison, WI, USA). The ligation mixture containing product from RT-PCR was transformed into *Escherichia coli* DH5-α competent cells. Transformants were selected by amplicilin LB-agar plates and the presence of the pGEM®-T easy containing the insert was determined by screening for blue/white colonies using IPTG and X-gal.

Analysis of gene expression by RT-PCR

Primers for RT-PCR were designed based on the VGP450-1 sequences. A forward primer, VGP450-1F= 5'-TTTGAACCAGAAAGGTTCATGGG-3') and a reverse primer, VGP450-1R= 5'-GTTGTCATGATCATAAACTTTAGCC-3'). RT-PCR using this primer generates a DNA fragment of about 270 bp.

Total RNA was prepared from the cultured cells using Trizol reagent (Invitrogen/Gibco). The RNA (5ng) was reverse-trascribed(RT) at 42 ^{o}C for 1 h using Superscript II reverse transcriptase (Invitrogen/Gibco) with 5X first strand RT buffer, 10 mM dithiotreitol, 2 mM each of dNTP and 0.1 mM oligo(dT) primer and 10 U of

RNase inhibitor in a total volume of 20 μl. A 1 μl aliquot was used for PCR with 0.5 U Taq polymerase, 1X buffer, 0.2 mM each dNTP and 10 μM forward and reverse primers. The amplification program consisted of one cycles of a 3-min denaturation at 94 ^{o}C; 28 cycles of 30-s denaturation at 94 ^{o}C, a 30-s primer annealing at 52 ^{o}C and a 30 -s elongation at 72 ^{o}C; one final elongation cycle 3 min at 72 ^{o}C. The amplified products were separated by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining.

DNA sequencing and analysis

All the DNA sequencing reaction were performed by the BigDye Terminator Cycle sequencing kit (Perkin-Elmer, Wellesley, MA), with the sequences being read on an automated ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Computer assessment of the DNA sequence information was performed using VectorNTI software package.

5'-Rapid amplification of cDNA ends (5'- RACE)

To obtain the 5'-terminal sequence of VGP450-1, 5'-RACE was carried out with a gene-specific primer at heme binding domain (5'-CCAGGACAAATTCTACGACCAGACCC-3'), using the GeneRacer kit (Invitrogen/Gibco), according to the manufacturer's manual. For this, total RNA was isolated from phenobarbital-treated *V. glabrata* cultured cells using Trizol Reagent (Invitrogen/Gibco) according to the manufacturer's instruction. Poly(A)$^{+}$ mRNA was purified from the total RNA using Oligotex resin (Qiagen, Santa Clarita, CA). Amplification of 5'-end of cDNA was performed according to the protocol described in the GeneRacer kit (Invitrogen/Gibco). Two hundred and fifty nanograms of mRNA was treated with calf intestinal alkaline phosphate to remove the 5'terminal phosphate from any RNA that is not full length mRNA. These RNA were then treated with tobacco acid pyrophosphatase to remove the 5'-cap structure of the full length mRNA thus leaving a 5'-PO4 structure of the full length mRNA. RNA ligase was then used to ligase the GeneRacer RNA-oligo primer to only the full length mRNA. First strand cDNA synthesis was accomplished with SuperScript III Reverse Transcriptase, GeneRacer Oligo (dT) primer, and RNA treated as described above as template in 20

μl reaction according to the manufacturer's protocol. Two microlitres of this reverse transcriptase reaction mixture was used as templates for second-stranded cDNA synthesis and amplification. PCR was performed at 94 ^{o}C for 2 min, followed by 25 cycles of 94 ^{o}C for 30 s, 65 ^{o}C for 10 s and 72 ^{o}C for 3 min, with an additional extention step at 72 ^{o}C for 10 min. The amplified PCR products were subcloned into pGEM-T easy vector and sequenced.

Results and discussion

20-hydroxyecdysone accumulation is induced by phenobarbital

Phenobarbital is known as a P450 inducer. It had been proved to effectively stimulate alkaloid biosynthesis in *Catharanthus roseus* cell culture (Contin *et al*., 1999; Zhao *et al*., 2000). Induction of plant cytochrome P450 enzymes by phenobfrffgarbital has also been reported (Simpson and Kelly 1989; Reichhardt *et al*., 1979; Reichhardt *et al*., 1980). The effect of the presence of cytochrome P450 inducer, phenobarbital was shown in Figure 2. The phenobarbital was tested at doses of 10 mg/L or 20 mg/L was observed to lead to an increase both cell growth and 20-hydroxyecdysone production. The significance of a phenobarbital effect on increasing 20-hydroxyecdysone levels was indicated at both doses and at 6 days, 8 days and 10 days. At 6 days the 20-hydroxyecdysone level increased approximately 7.5 fold in comparison to control cells. Phenobarbital, a potent inducer of xenobiotic-metabolizing monooxygenases, was a good inducer of microsomal ecdysone 20-monooxygenase in fall armyworm larvae. Phenobarbital, a well-known cytochome P450 inducer, also caused a 2-fold increase in the microsomal ecdysone 20-monooxygenase activity of fall armyworm, *Spodoptera frugiperda* (Yu, 1995). This compound also stimulated the ecdysone 20-monooxygenase activity in the African migratory locust as judged by the in vivo conversion of ecdysone to 20-hydroxyecdysone (Feyereisen and Hoffmann, 1977). However, in house fly larvae, Phenobarbital had no effects on ecdysone 20-monooxygenase activity in microsomes, but depressed it in mitochondria (Agosin *et al*., 1988). Keogh *et al*. (1989) also reported that phenobarbital did not stimulated midgut ecdysone 20-monooxygenase activity in tobacco hornworm laevae.

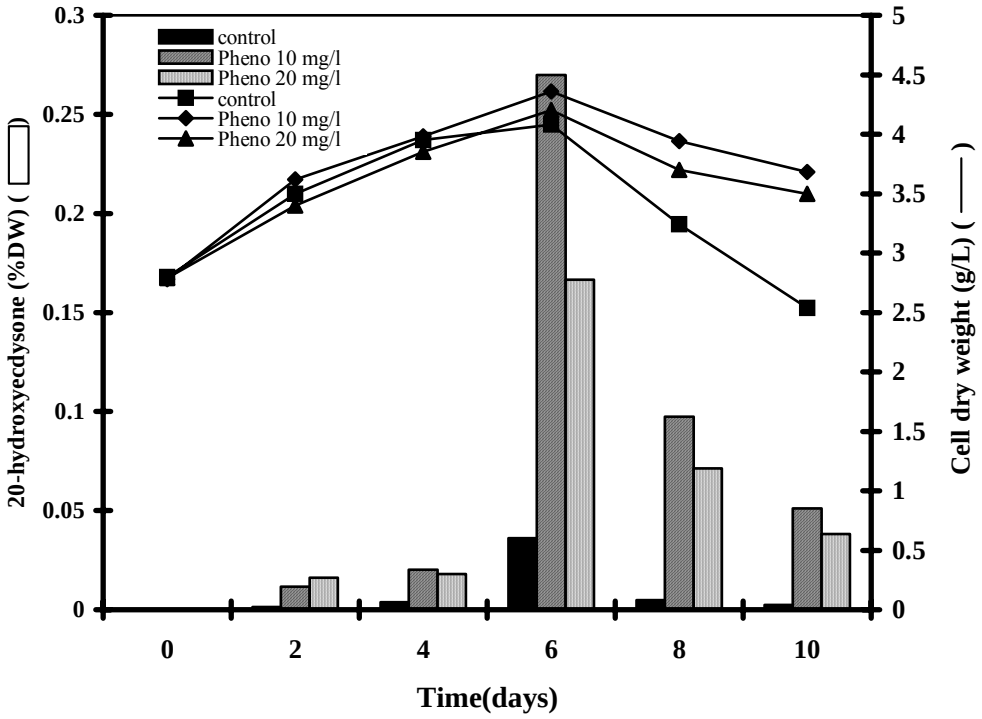


Figure 2 Effect of phenobarbital treatment on cell growth and 20-hydroxyecdysone accumulation in *V. glabrata* cultured cells.

Isolation of phenobarbital-inducible cytochrome P450 cDNA from *V. glabrata* suspension-cultures cells

The above results suggesting that phenobarbital induced 20-hydroxyecdysone accumulation. To isolate candidate P450 cDNAs using PCR-based strategy, we prepared the mRNAs from phenobarbital-treated *V. glabrata* cell culture. Sequence alignments of insect known ecdysone 20-monooxygenase were used to identify conserved regions to which a series of degenerate primers were prepared (Figure 3 and Table 1).

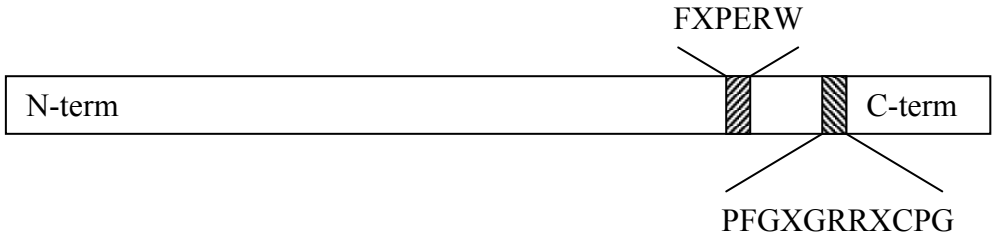


Figure 3 A schematic diagram of the primary structure of known insect ecdysone 20-monooxygenase with conserved domains used for the design of PCR primer highlighted.

Table 1 Degenerate P450-specific primer for PCR cloning strategy.

Primer name	Sequence
FXPER(F/W)	5'-TTYCTICCGGAGCGDTKB-3'
PFGXGRRX	5'-CCVTTCGGMVBBGGIMGVAGD-3'
GRRXCP(A/G)	5'-GGIMGIMGIIIITGYCCIGS-3'

Products resulting from the PCR using the FXPERW primer, which displayed bands of ca. 550 bp (Figure 4), were subcloned into a pGEM-T easy vector and randomly sequenced. The sequenced VGP450-1 had only 460 nucleotides and encoded a partial 3'-fragment of P450 cDNA. This clones containing conserved FXPERF motif and PFGXGRRXCPG motif were assumed to be fragments of P450 cDNA (Figure 5).

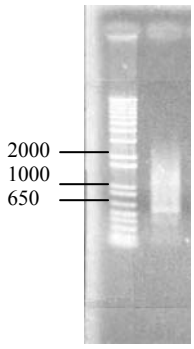


Figure 4 Ethidium bromide-stained agarose gel showing the PCR products amplified from a cDNA prepared with mRNA isolated from phenobarbital-treated cells using the degenerate FXPER(F/W).

AGA GTG GTT GAG CTC TAC TTA TGG GCA GTA GGG CGA GAC CCT 42
R V V E L Y L W A V G R D P ***14***
AAC TGC TGG GAA AGA CCT GAA GAG TTT GAA CCA GAA AGG TTC 84
N C W E R P E E ***F E P E R F*** ***28***
ATG GGT AGT GGC AGT AGT ATT GAT TAC AAA GGG ACA GAT TTT 126
M G S G S S I D Y K G T D F ***42***
GAG TTG ATC CCA TTT GGG TCT GGT CGT AGA ATT TGT CCT GGA 168
E L I ***P F G S G R R I C P G*** ***56***
ATA CCG ATG GCG GCT GTA ACA ATG GAA ATG ACT CCT TCA AAT 210
I P M A A V T M E M T P S N ***70***
CTT ATT TAC TCG TTT GAT TGG GAA TCA CCT GTG AAA GCA CAT 252
L I Y S F D W E S P V K A H ***84***
GAA ATT GAT ACC TCA AAA TCT CCT GGT GCG GTA ACA CAT AAG 294
E I D T S K S P G A V T H K ***98***
AAG AAT GCA CTC CAA CTA ATG GCT AAA GTT TAT GAT CAT GAC 336
K N A L Q L M A K V Y D H D ***112***
AAC AAG AAC TAA tgagaatcctaacttctatgcattcttatttcttaccatttctaataattatgtatg
N K N *
ttttg aaaattgttatactattcgagttataaaaaaaaaaaaaa

Figure 5 Nucleotide sequence and deduced amino acid sequence of VGP450-1 cDNA isolated from phenobarbital-treated *V. glabrata* cell culture. The heme-binding domain is indicated with underlines. Star denotes translational stop codon.

Sequence comparison of P450 fragment

BLAST searches in the GenBank database revealed that the VGP450-1 cDNA fragment encoded for protein shows a relatively high degree of sequence homology to cytochrome P450 protein from a variety of plant species. (Table 2). The deduced amino acid sequence of VGP450-1 shared the highest identity (60%) with that of CYP83G1 of *Medicago truncatula*.

Table 2 Sequence alignment of VGP450-1 with other plant P450s

CYP	Species	Percentage amino acid identity
CYP83G2	*Medicago truncatula*	59%
CYP83G1	*Medicago truncatula*	60%
CYP83H1	*Medicago truncatula*	53%
CYP83H2	*Medicago truncatula*	54%
CYP83E8	*Glycine max*	52%

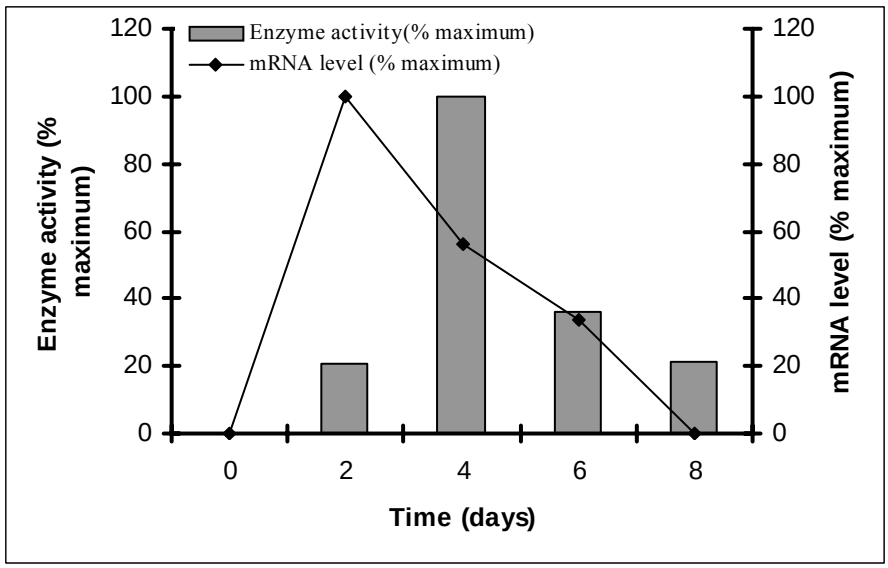
Alignment of the amino acid sequences of VGP450-1 with those of the reported ecdysone 20-monooxygenase gene in insect species was done (Table 3).

Table 3 Sequence alignment of VGP450-1 with other insect known CYP314A1 in various species

Species	Percentage amino acid identity
Drosophila melanogaster	22%
Anopheles gambiae	13%
Aedes aegypti	15%
Manduca sexta	19%
Bombyx mori	18%
Apis mellifera	18%

Phenobarbital-treated changes of VGP450-1 transcript level , ecdysone 20-monooxygenase, and 20-hydroxyecdysone production

Because it was not possible with the above information to ascribe a particular function of VGP450-1, we hypothesized that perhaps the VGP450-1 gene involved in 20-hydroxyecdysone metabolism. To get insight into the potential regulatory role of VGP450-1 in 20-hydroxyecdysone biosynthesis, we investigated the expression of the VGP450-1 gene in relation to ecdysone 20-monooxygenase and 20-hydroxyecdysone accumulation in cell cultured of phenobarbital-treated *V. glabrata*. The period of high transcript levels correlated well with the time of increasing enzyme activity (Figure 6A). RT-PCR analysis showed that the VGP450-1 transcript level were correlated with ecdysone 20-monooxygenase activity and 20-hydroxyecdysone production, suggesting that 20-hydroxyecdysone biosynthesis is regulated at the transcriptional level of the VGP450-1 gene. In order to characterize the relationship between ecdysone 20-monooxygenase activity and 20-hydroxyecdysone biosynthesis, we analysed the changes in ecdysone 20-monooxygenase activity and in 20-hydroxyecdysone accumulation (Figure 6B). 20-hydroxyecdysone in *V. glabrata* cells started to increase gradually within 2-4 days after phenobarbital addition, followed by a rapid increase until 6 days. Ecdysone 20-monooxygenase activity rose within 2 days after phenobarbital treatment, reached a peak at 4 days and thereafter decreased, consistent with the phenobarbital-induced changes in 20-hydroxyecdysone production. RT-PCR analysis demonstrated a mRNA induction time course consistant with the induction of the VGP450-1 mRNA translational activity. VGP450-1 mRNA was not detected in control cells (Figure 7).



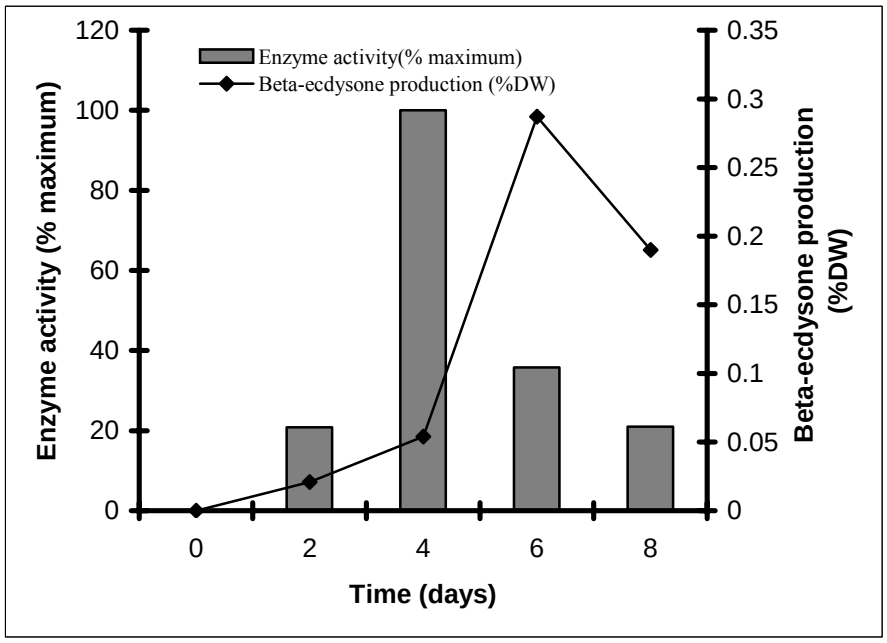


Figure 6 Induction time course for ecdysone 20-monooxygenase, VG-P450-1 expression and 20-hydroxyecdysone production. A) Time course of phenobarbital-induced VGP450-1 mRNA accumulation and enzyme activity. VGP450-1 mRNA levels were quantified by Image J 1.366 program. B) Time course of ecdysone 20-monooxygenase activity and 20-hydroxyecdysone accumulation in phenobarbital-induced *V. glabrata* cells.

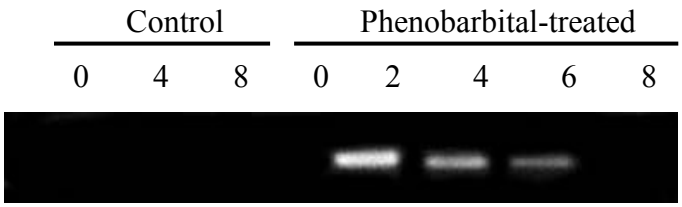


Figure 7 RT-PCR analysis of mRNA levels of VGP450-1 in *V. glabrata* cell suspension cultures after the addition of phenobarbital. The suspension cultures were supplemented with Phenobarbital at a final concentration of 10 mg/L and the cells were collected for analysis 0, 2, 4, 6 and 8 days after phenobarbital addition.

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RESEARCH OUTPUT

PUBLISHCATIONS IN PROCEEDING AND ABSTRACTS

1. Sinlaparaya D, Panichajakul. **Effect of Ecdysteroids Precursors Feeding on Formation of Beta-ecdysone in Cell Suspension Cultures of *Vitex glabrata* R.Br. (Poster presentation).** Proceeding of the Biothailand 2001: From Research to Market. 7-10 November 2001. Queen Sirikit National Convention Center, Bangkok, Thailand, 291.

2. Sinlaparaya D, Panichajakul. **Selective Cloning and Determination of the Ecdysone 20-monooxygenase in *Vitex glabrata* R.Br. Cell Suspension Cultures by High-performance Liquid Chromatography. (Oral presentation)** Proceeding of the 15th Annual Meeting of the Thai Society for Biotechnology: Sustainable Development of SMEs Through Biotechnology and JSPS-NRCT Symposium: The Forefront of Bioinformatic Application. 3-6 February 2004. Pang Suan Keaw, Chiang Mai, Thailand, 40.

3. Sinlaparaya D, Panichajakul. **Selective Cloning and Determination of the Ecdysone 20-monooxygenase in *Vitex glabrata* R.Br. Cell Suspension Cultures by High-performance Liquid Chromatography. (Poster presentation)** 20-23 April 2005. TerpNet 2005: Wageningen, The Netherlands, 132.

APPENDIX

MANUSCRIPT

I. Optimization of Growth and 20-Hydroxyecdysone Production of *Vitex glabrata* R.Br. Cell Suspension Culture

II. Enhancement of 20-hydroxyecdysone production in cell suspension cultures of *Vitex glabrata* R.Br. by precursors feeding

I. Optimization of Growth and 20-Hydroxyecdysone Production of *Vitex glabrata* R.Br. Cell Suspension Culture

(This manuscript submitted in the Chinese Journal of Biotechnology)

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Abstract

The effects of the cultivation media, plant growth regulators and inoculum size on the growth and 20-hydroxyecdysone production of suspension cultures of *Vitex glabrata* R.Br. were investigated. The cell growth and 20-hydroxyecdysone formation reach the highest when cells were cultured in the Gamborg's B5 medium supplemented with 2.0 mg/L BAP and 1.0 mg/L 2,4-D. The maximum 20-hydroxyecdysone productivity, of about 1.1 mg/L/day, was observed in the culture with 20 %PCV of inoculum size. These data also show that increment of the inoculum size to 20 %PCV could increase 7-folds of productivity.

Keywords : 20-hydroxyecdysone, *Vitex glabrata*, suspension cultures

Introduction

The ecdysteroid 20-hydroxyecdysone or beta-ecdysone, is one of the moulting hormone which plays a key role in growth, development and reproduction of insects and crustaceans[1]. Ecdysteroids and analogues could be use as insecticides[2]. In addition, beta-ecdysone has the potential to substitute supplementary cholesterol and serve as a natural growth stimulant in shrimp feeds[3]. Recent studies on the pharmaceutical properties of 20-hydroxyecdysone, showed it as antimicrobial activity, antidiabetic effect, antioxidative and anti-free radical properties[4]. Many product containing 20-hydroxyecdysone for body-bluilders and sportmen can be found on the market. Levels of 20-hydroxyecdysone range as high as 2-2.5 %DW from barks of *V. glabrata* was supported the potential of using this plant as a source of moulting hormone[5]. However, the 20-hydroxyecdysone supply is very limited. It is because of scarcity and slow growth of *V. glabrata* trees. Plant cell culture has been suggested as an attractive alternative that could overcome the limitations of extracting useful metabolites from natural resources[6]. Many factors affecting 20-hydroxyecdysone production from *V. glabrata* cultured cells have been investigated, including medium optimization [5,7], precursors feeding[7]. The objectives of this work were to establish cell suspension cultures of *V. glabrata* for the 20-hydroxyecdysone production by manipulating the different medium formulations and type of hormone. The effect of inoculum size on cell growth and 20-hydroxyecdysone production was also studied.

1 Materials and methods

1.1 Plant material and cell cultures

V. glabrata cells lines was initiated from young stem as previously described [5], which was subcultured for 10 years. Callus culture were maintain in half strength MS (Murashige and Skoog) medium[8] supplemented with 2.0 mg/L 6-benzylaminopurine (BAP), 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 30 g/L sucrose. The medium was adjusted to pH 5.8 and then sterized by autoclaving at 121 ^{o}C for 20 min. Suspension cultures were cultured in a liquid medium similar to that for callus culture, but excluding agar. Flasks were placed on a rotary shaker at 120 rpm under continuous light at 2000 lux at 25 ^{o}C.

1.2 Cell growth determination

Cell growth was determined by measuring the increase in the cell dry weight of the culture. For the determination of cell dry weight, cell suspensions were filtered through filter paper on the Buchner funnel under vacuum. The cell were placed on petridish and dried in an oven at 60 ^{o}C for about 2 h to constant weight.

1.3 Ecdysteroid extraction and HPLC analysis

A 0.3 g mass of dry cells were extracted with 180 ml of 95% (v/v) ethanol in a soxhlet apparatus for 6 h. The ethanol extracts were evaporated by rotary evaporator at 60 ^{o}C. The residue was dissolved in 3 ml methanol and then vortexed with 5 ml hexane for twice. The methanol extracts were evaporated at 60 ^{o}C in hot air oven. The residue was dissolved in 2 ml distilled water. The supernatant was filtered through Sep-pak C_{18} cartridge. Highly polar material was separated from the retained ecdysteroid fraction by elution with 10 ml distilled water. Ecdysteroids were eluted from the cartridge with 20 % (v/v) methanol-water (10 ml) and 80 % (v/v) methanol-water (10 ml), respectively. The elution was collected and dried at room temperature and dissolved in methanol for HPLC analysis. The extracts were analyzed using ODS-3 C18 column (250x4.6mm). The mobile phase consisted of water/acetonitrile/acetic acid (84:14:2, v/v/v) at 1.0 ml/min. The absorbance was measured at a wavelength of 254 nm.

2 Results and discussion

2.1 Effect of cultivation media

Fig. 1 displays the time courses of cell growth and 20-hydroxyecdysone production in suspension cultures in B5 medium and 1/2MS medium, both supplemented with 2.0 mg/L BAP and 1.0 mg/L 2,4-D. The highest cell dry weight of 12.1 g/L was attained in B5 medium on week 3. In the 1/2MS medium, the maximum cell dry weight was found lower than B5 medium by about 20%. HPLC analysis of 20-hydroxyecdysone showed that cells grown on B5 medium produced maximum 20-hydroxyecdysone (0.038 %DW) in the third week of cultivation. This value was 24% higher than that using 1/2MS medium. According to the report of Nahalka and co-worker, the plumbagin content in the B5 medium was about 1.8 fold higher than that in the 1/2MS medium[9]. B5 medium was more effective for the cell growth and 20-hydroxyecdysone formation probably because

it had more inorganic nutrients and vitamins than MS medium[10]. Moreover, B5 medium also was optimal for cell growth and secondary metabolites formation in other plant, including *Crocus sativa* [11] and *Cistanche deserticola* [12]. In the present work, B5 medium was effective in promoting both cell growth and 20-hydroxyecdysone production. Hence in this work, B5 medium was chosen as the medium to undergo further investigations.

2.2 Effect of plant growth regulators

Effect of plant growth regulators on the cell growth and 20-hydroxyecdysone production of *V. glabrata* cell suspension cultures was also studied (Fig. 2). *V. glabrata* cells were cultivated in the B5 medium supplemented with 2.0 mg/L BAP and 1.0 mg/L of 2 different auxin (2,4-D and IAA). The cell growth in B5 medium supplemented with 2.0 mg/L BAP and 1.0 mg/L IAA reached the maximum growth on week 4 about 7.4 g/L. Whereas, in B5 medium supplemented with 2.0 mg/L BAP and 1.0 mg/L 2,4-D, the maximum cell growth was reached on week 3, 40% higher than that in the medium containing IAA. The maximum 20-hydroxyecdysone content was 0.039 %DW in B5 medium supplement with 2.0 mg/L BAP and 1.0 mg/L 2,4-D on week 3 of cultivation. This content was 30% greater than that in medium with IAA. Accordingly, B5 medium supplemented with 2.0 mg/L BAP and 1.0 mg/L 2,4-D was used in the further experiment.

2.3 Effect of inoculum size

The effect of inoculum size on cell growth and 20-hydroxyecdysone production is given in Table 1. The highest cell growth at 20 % Packed Cell Volume (PCV) of inoculum size was obtained at day 4 of cultivation while cells cultured at 10 %PCV reached on day 21. An increase in inoculum size greatly shortened the cultivation time. The highest 20-hydroxyecdysone formation of about 0.040 %DW was found in the inoculum size of 20 %PCV at 4 days of cultivation. The maximum productivity of 20-hydroxyecdysone was 1.10 mg/L/day at 20 %PCV. This value was 7 folds greater than that using 10 %PCV. This indicates that an increase in cell inoculum size is important for increasing 20-hydroxyecdysone production. The stimulation of ginseng saponin production by increased inoculum size may be due to cell-to-cell communication and unknown factors

released by inoculum size[13]. Moreover, the effect of inoculum size on the cell growth and secondary metabolite production may be related to the enhancement of the activity of the enzymes involved in the metabolic pathway[14]. In the same way, the increase in inoculum size, in order to achieve higher levels of secondary metabolites including taxol for *Taxus chinensis* [15], cucurbitacin for *Cucurbita andreana*[16], ginseng saponin for *Panax notoginseng* [17] and jaceosidin for *Saussurea medusa*[18]. However, it is still unclear about the detailed mechanism of inoculum size effect on the metabolite biosynthesis.

3. Conclusion

In conclusion, the optimum conditions for the cell growth and 20-hydroxyecdysone production of the cell suspension cultures of *V. glabrata* were in the Gamborg's B5 medium supplemented with 1.0 mg/L 2,4-D, 2.0 mg/L BAP and 20 %PCV of inoculum size were established.

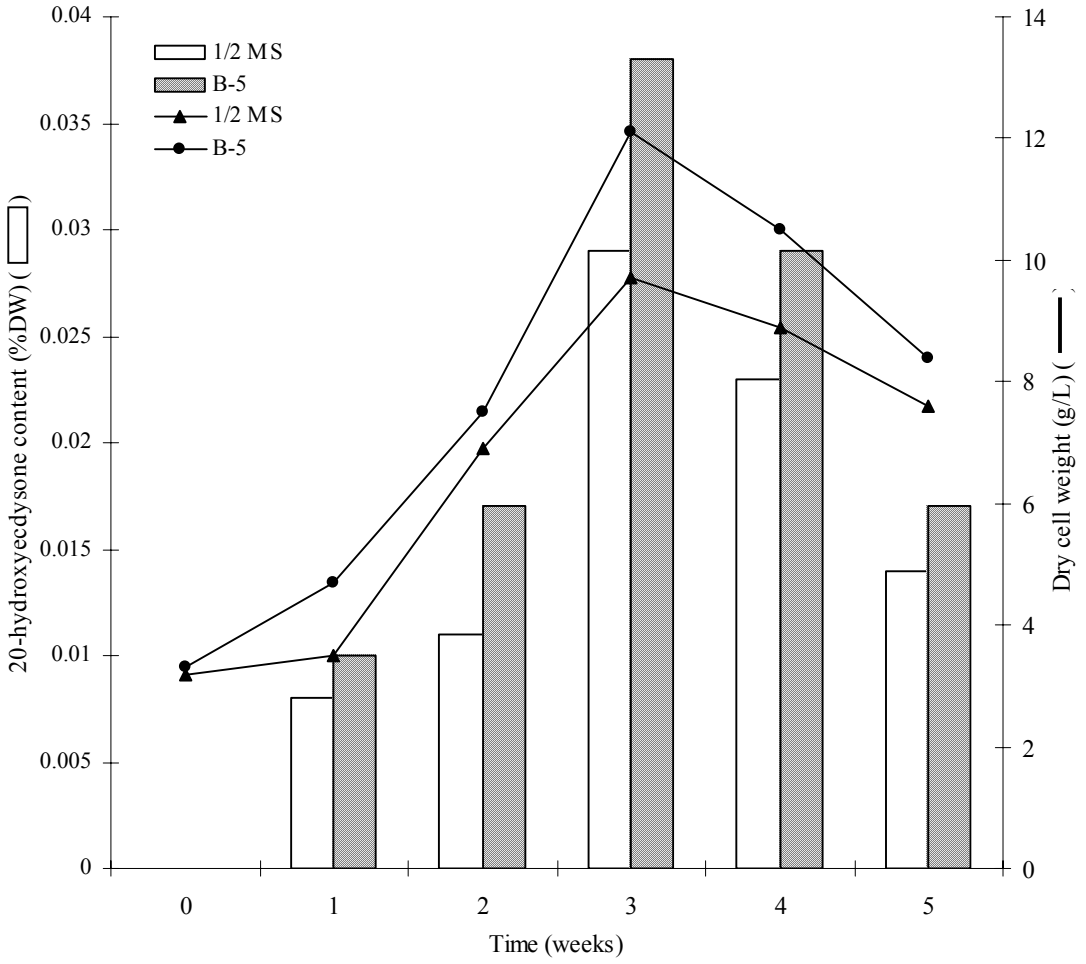

Figure 1 Time course of dry weight and 20-hydroxyecdysone production of *V. glabrata* cell suspension cultures in 2 different media formulation.

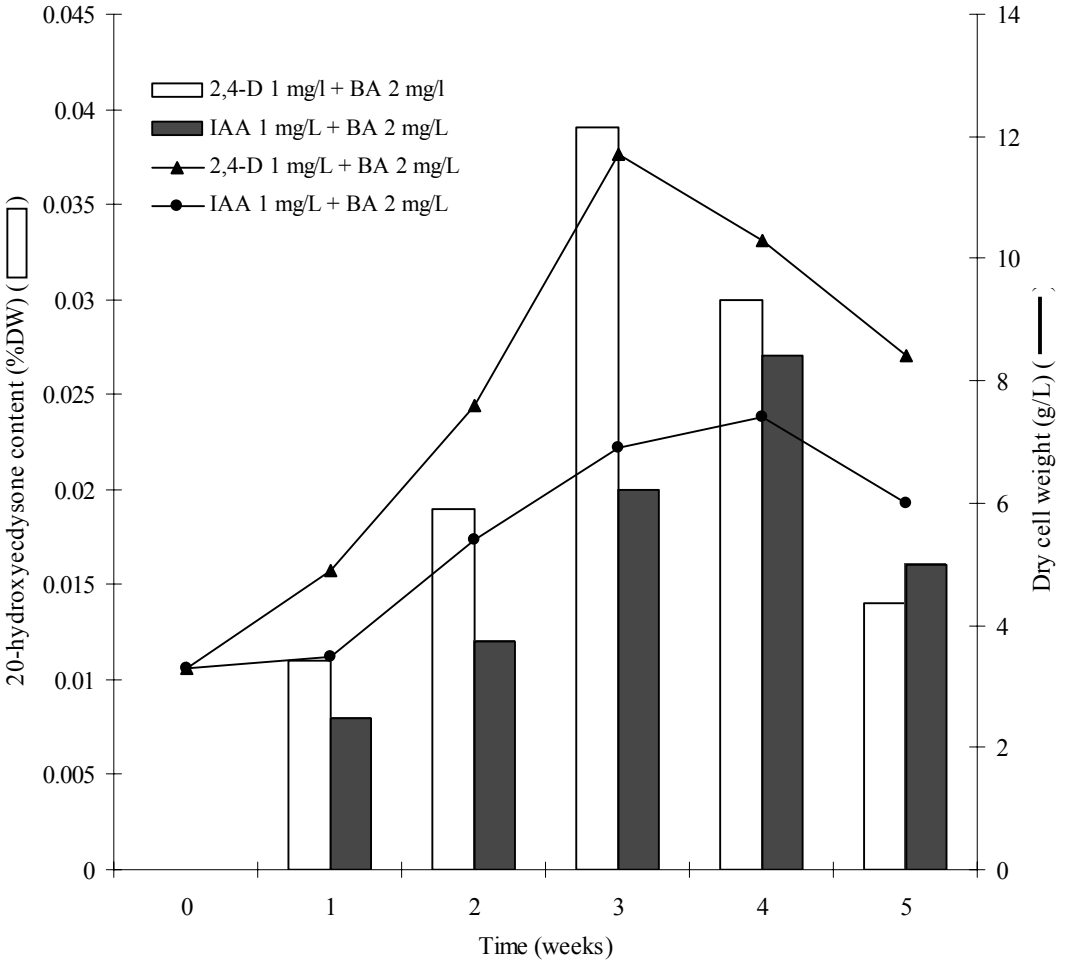

Figure 2 Time course of dry weight and 20-hydroxyecdysone production of *V. glabrata* cell suspension cultures in B5 medium supplement with 2.0 mg/L BAP and 1.0 mg/L of 2 different auxin (2,4-D and IAA).

Table 1 Effect of inoculum size on the biomass and 20-hydroxyecdysone productivity in suspension culture of *V. glabrata* in B5 medium supplemented with 2.0 mg/L BAP and 1.0 mg/L 2,4-D.

Inoculum size (%PCV)	Maximum production (%DW)	Maximum cell growth (g/L)	Time of maximum production (day)	Maximum productivity (mg/L/day)
10	0.033	11.2	21	0.176
20	0.040	11.0	4	1.1

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II. Enhancement of 20-hydroxyecdysone production in cell suspension cultures of *Vitex glabrata* R.Br. by precursors feeding

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Abstract

The effect of ecdysteroid precursors feeding on cell growth and 20-hydroxyecdysone production of *Vitex glabrata* suspension cultures were studied. The addition of cholesterol, there was no appearance increased of 20-hydroxyecdysone contents while partially inhibited growth at higher levels. Feeding of 7-dehydrocholesterol and ergosterol did not affect the cell growth. Both precursors effectively increased production of 20-hydroxyecdysone. Feeding of 7-dehydrocholesterol as a precursor was most effective. The maximum 20-hydroxyecdysone productivity of about 1.31 mg/L/day was observed in the culture with 10 mg/L 7-dehydrocholesterol. This data is the first indication that 7-dehydrocholesterol and ergosterol feeding are an effective precursors for 20-hydroxyecdysone formation in plant cell suspension culture.

Keywords: 20-hydroxyecdysone, precursor feeding, suspension cultures, *Vitex glabrata*

INTRODUCTION

The ecdysteroid 20-hydroxyecdysone, is the steroid hormone of arthropods, which plays a crucial role in molting, metamorphosis, reproduction and diapause (Butenandt and Karlson, 1954). Ecdysteroids and analogues could be use as insecticides (Dhadialla and Tzertzinis, 1998). Moreover, it has been used in the shrimp culture in order to increase productivity (Chaiwatcharakool, 1986). 20-hydroxyecdysone and derivatives were prepared for health improvement, they have been shown to stimulate the synthesis of protein, builds muscle, adaptogenic for HIV patients, antioxidant and tonics properties (Bathori, 2002). The ready availability of large amount of 20-hydroxyecdysone from plant sources had led to boom in recent years in its inclusion in many commercial anabolic preparations for body-builders and sportmen (Dinan and Lafront, 2006). Typically, insects acquire plant sterols like campesterol and convert the plant sterol into 20-hydroxyecdysone. However, some plants accumulate ecdysteroids constitutively in what is thought to be a premature defense response that could serve to induce premature developmental changes and death if ingestion by chewing insects. Accumulation of 20-hydroxyecdysone has been reported for several plants, but is especially high in the bark of *V. glabrata* (Werawattanametin, 1986; Thavornnithi, 1990). The biosynthesis of 20-hydroxyecdysteroid by insects has been established, but little is currently known about this capacity in plants. Studies using plant cell cultures are also of interest for the study of phytoecdysteroid biosynthesis. Many strategies have been followed to increase 20-hydroxyecdysone production from *V. glabrata* cell culture including, medium optimisation (Thavornnithi, 1990, Prasertsom, 1990), precursor feeding (Prasertsom, 1990) and cell line selection (Duanghaklang, 2001). The objective of this study was to investigate the effect of precursors feeding on cell growth and 20-hydroxyecdysone production of *V. glabrata* suspension cultures. In this study, the effect of precursor feeding on the biosynthesis of 20-hydroxyecdysone in the plant cell suspension culture were report for the first time.

MATERIALS AND METHODS

Plant material and culture method

V. glabrata cells, initially induced from stem and subcultured for over 10 years (Thavornnithi, 1990). Callus culture were incubated on the growth solid medium, which is half strength MS medium supplemented with 2.0 mg/L 6-benzylaminopurine (BAP), 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g/L sucrose and 8 g/L agar. Suspension culture for 20-hydroxyecdysone production, the cells were transferred to the production medium, Gamborg's B5 medium supplemented with 2.0 mg/L BAP and 1.0 mg/L 2,4-D. Flasks were placed on a rotary shaker at 120 rpm under continuous light at 2000 lux at 25 ^{o}C.

Addition of precursors

Sterized precursors including cholesterol (100 and 200 mg/L), 7-dehydrocholesterol (1 and 10 mg/L) and ergosterol (1 and 10 mg/L), were added to the cell cultures on the day of inoculation. Cells were cultured for several days and harvested for analysis of 20-hydroxyecdysone content and biomass yield. All treatment were performed in duplicate. The cells of each treatment were harvested after 12, 24, 48, 72, 96 and 120 hrs.

Determination of dry cell weight

Cell growth was determined by measuring the increase in the cell dry weight of the culture. For the determination of cell dry weight, cell suspensions were filtered through filter paper on the funnel under vacuum. The cell were placed on petridish and dried in an oven at 60 ^{o}C for about 2 h to constant weight.

Ecdysteroid extraction and analysis

20-hydroxyecdysone was extracted from dried cells as described by Duanghaklang (2001). A 0.3 g mass of dried cells were extracted with 95% ethanol (180 ml) in a soxhlet apparatus for 6 h. The ethanol extracts were evaporated by rotary evaporator at 60 ^{o}C. The residue was dissolved in 3 ml methanol and vortexed with 2 ml hexane for twice. The methanol extracts were evaporated at 60 ^{o}C in hot air oven. The residue was dissolved in 2 ml distilled water. The supernatant was filtered through Sep-pak C_{18}

cartridge. Highly polar material was separated from the retained ecdysteriod fraction by elution with 10 ml distilled water. Ecdysteroids were eluted from the cartridge with 20 %(v/v) methanol-water (10 ml) and 80 %(v/v) methanol-water (10 ml), respectively. The elution was collected and dried at room temperature and dissolved in methanol for HPLC analysis. 20-hydroxyecdysone was analyzed by HPLC using reverse phase C18 column with detection at 254 nm. The elution was performed isocratic gradient with 14% acetonitrile in 2% acetic acid. The elution rate was kept at 1.0 ml/min.

RESULT AND DISCUSSION

Effect of cholesterol feeding on cell growth and 20-hydroxyecdysone production

The effect of cholesterol feeding on cell growth and 20-hydroxyecdysone of *V. glabrata* suspension culture was shown in Figure 1. The feeding of cholesterol (100 and 200 mg/L) to the cell cultures of *V. glabrata* significantly decreased the growth of the cells over the control culture. It was observed that cholesterol, the early biosynthetic precursor of ecdysteroid pathway, did not increase 20-hydroxyecdysone production. Similarly, Prasertsom (1990) reported that no increase in 20-hydroxyecdysone production in *V. glabrata* cell culture when cholesterol feeding at a concentration 100-200 mg/L. Cholesterol feeding did not enhance the 20-hydroxecdysone production, probably due to too high level of cholesterol.

Effect of 7-dehydrocholesterol feeding on cell growth and 20-hydroxyecdysone production

Figure 2 shows the effect of 7-dehydrocholesterol on cell growth and 20-hydroxyecdysone production of *V. glabrata* suspension cultures. No significant variation in cell growth was observed when the 7-dehydrocholesterol level was varied in the range of 1 and 10 mg/L. The addition of 7-dehydrocholesterol to the cell cultures of *V. glabrata* improved the production of 20-hydroxyecdysone. The maximum amount of 20-hydroxyecdysone was found to be 0.045 %DW on 96 h using 10 mg/L 7-dehydrocholesterol. The increased was about 1.36-fold over the control cultures. This results suggested that improvement of 20-hydroxyecdysone by 7-dehydrocholesterol feeding may be due to its incorporation as a precursors for the biosynthesis of 20-

hydroxyecdysone. The earlier reports suggest that 7-dehydrocholesterol is the biosynthetic precursor of ecdysteroid in plant (Ohyama et al., 1999). In addition, Grieneisen et al. (1994) reported that radiolabelled 7-dehydrocholesterol was incorporated into 20-hydroxyecdysone in insects.

Effect of ergosterol feeding on cell growth and 20-hydroxyecdysone production

As shown in Figure 3, effect of ergosterol feeding on the cell growth and 20-hydroxyecdysone production of *V. glabrata* cell suspension cultures was also studied. The feeding of ergosterol did not affect the biomass production of the cell culture. The maximum amount of 20-hydroxyecdysone was found to be 0.037 %DW on 96 h using 10 mg/L ergosterol. It was 1.12-fold increase in 20-hydroxyecdysone content over the control. This result is the first report that ergosterol feeding can enhance the 20-hydroxyecdysone content.

In conclusion, cholesterol feeding did not result in any increase in 20-hydroxyecdysone contents, while inhibiting cell growth in *V. glabrata* cell cultures. 7-dehydrocholesterol and ergosterol feeding led to increase 20-hydroxyecdysone production without affecting cell growth in *V. glabrata* cell cultures. The overall improvement in the yield of 20-hydroxyecdysone may be due to its incorporation as precursor for the biosynthetic pathway of 20-hydroxyecdysone.This results indicated the possibility of 7-dehydrocholesterol and ergosterol as the natural precursor for ecdysteroids biosynthetic pathway in plant cells. However, there is a need to study further employing the radiolabeled precursors to show the effect.

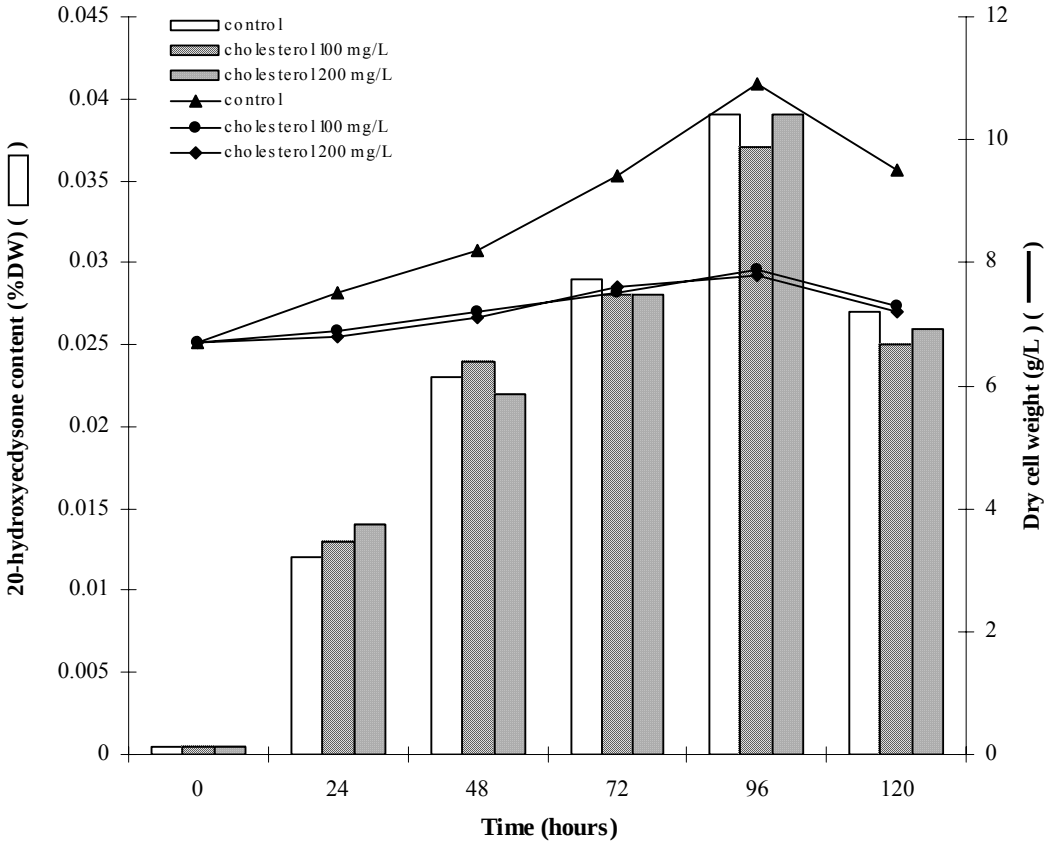

Figure 1 Effect of cholesterol feeding on cell growth and 20-hydroxyecdysone production of *V. glabrata* cell suspension cultures.

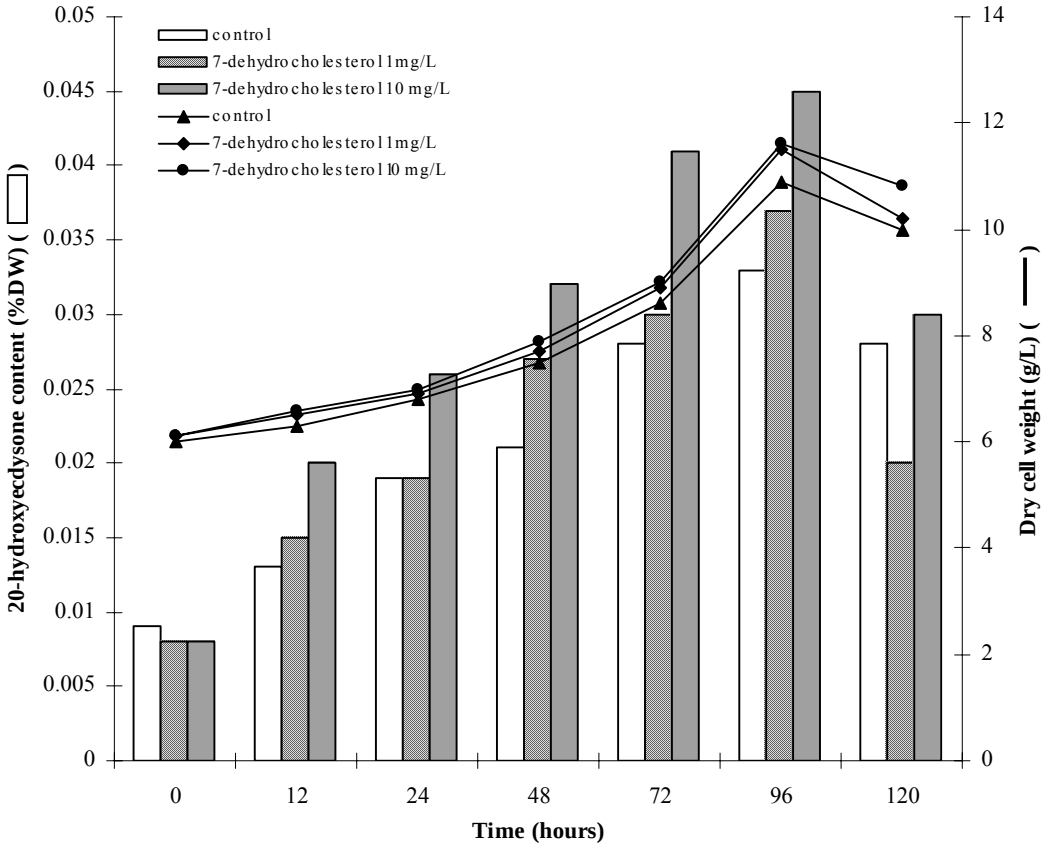

Figure 2 Effect of 7-dehydrocholesterol feeding on cell growth and 20-hydroxyecdysone production of *V. glabrata* cell suspension cultures.

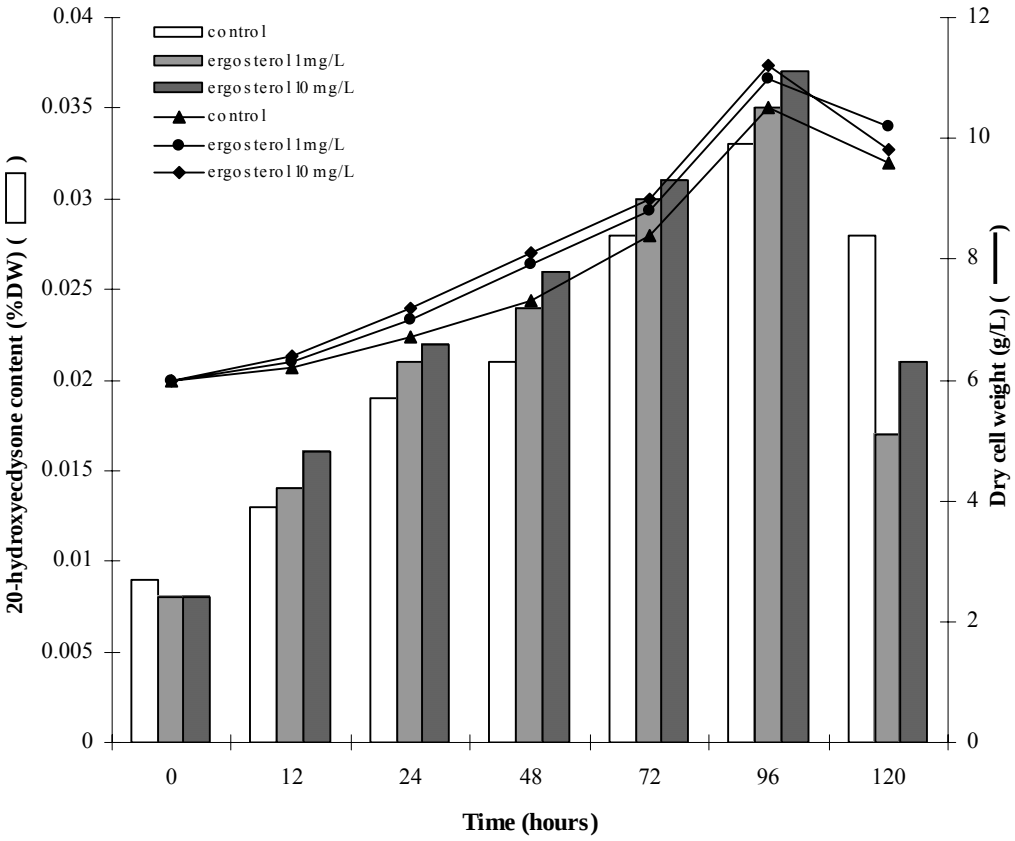

Figure 3 Effect of ergosterol feeding on cell growth and 20-hydroxyecdysone production of *V. glabrata* cell suspension cultures

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