

Full Length Research Paper

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

Duangjai Sinlaparaya, Preeyada Duanghakang and Sanha Panichajakul*

Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen, 40002, Thailand

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The effect of ecdysteroid precursors feeding on cell growth and 20-hydroxyecdysone production of *Vitex glabrata* suspension cultures were studied. On the addition of cholesterol, there was no apparent increase of 20-hydroxyecdysone while growth was partially inhibited 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 culture with 10 mg/L 7-dehydrocholesterol. This data is the first indication that 7-dehydrocholesterol and ergosterol feeding are effective precursors for 20-hydroxyecdysone formation in plant cell suspension culture.

Key words: 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 used 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, build muscle, be adaptogenic for HIV patients, have antioxidant and tonic 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 ingested by chewing insects. Accumulation of 20-hydroxyecdysone has been reported for several plants, but is especially high in the bark of *Vitex 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 optimization (Thavornnithi, 1990; Prasertsom, 1990), precursor feeding (Prasertsom, 1990) and cell line selection (Duanghakang, 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

*Corresponding author.
spanichajakul@yahoo.com

Email:

feeding on the biosynthesis of 20-hydroxyecdysone in plant cell suspension culture was reported for the first time.

MATERIALS AND METHODS

Plant material and culture method

V. glabrata cells, initially induced from stem was 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. For suspension culture of 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 of 2000 lux at 25°C.

Addition of precursors

Sterilized 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 was performed in duplicate. The cells of each treatment were harvested after 12, 24, 48, 72, 96 and 120 h.

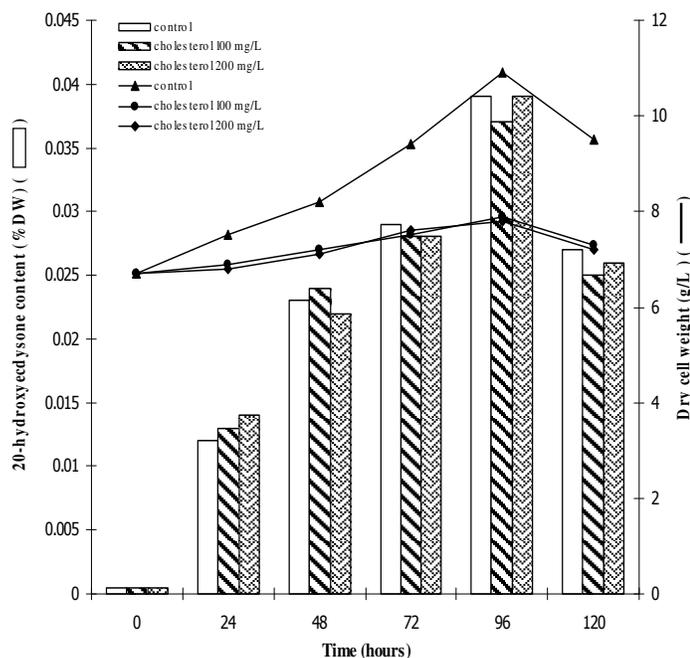


Figure 1. Effect of cholesterol feeding on cell growth and 20-hydroxyecdysone production in *V. glabrata* cell suspension cultures.

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 Petri dish and dried in an oven at 60°C for about 2 h to a 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 was extracted with 95% ethanol (180 ml) in a soxhlet apparatus for 6 h. The ethanol extracts were evaporated by rotary evaporator at 60°C. The residue was dissolved in 3 ml methanol and vortexed with 2 ml hexane twice. The methanol extracts were evaporated at 60°C in hot air oven. The residue was dissolved in 2 ml distilled water. The supernatant was filtered through Sep-pak C₁₈ 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. 20-hydroxyecdysone was analyzed by HPLC using reverse phase C18 column with detection at 254 nm. The elution was performed with isocratic gradient of 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 is 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 was observed when cholesterol was fed at a concentration of 100-200 mg/L. Cholesterol feeding did not enhance the 20-hydroxyecdysone 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.

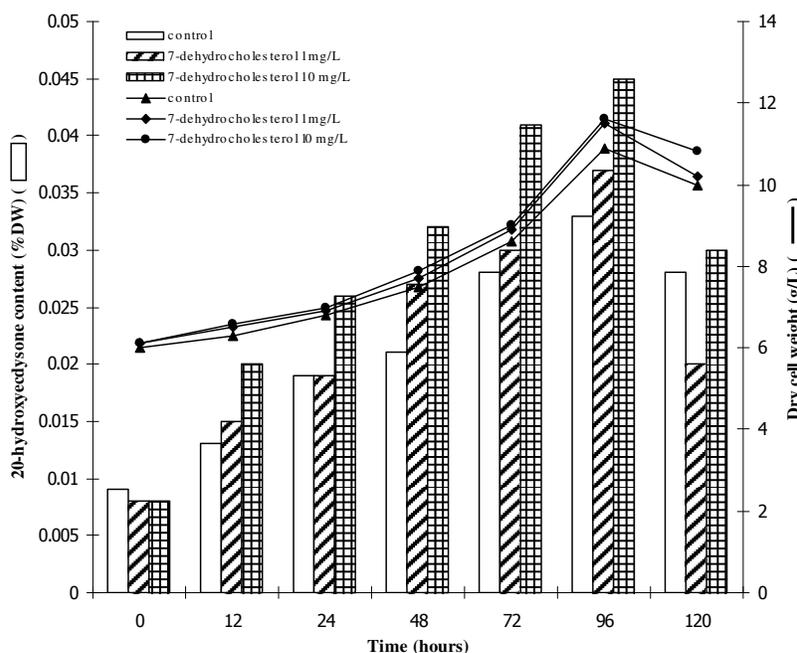


Figure 2. Effect of 7-dehydrocholesterol feeding on cell growth and 20-hydroxyecdysone production in *V. glabrata* cell suspension cultures.

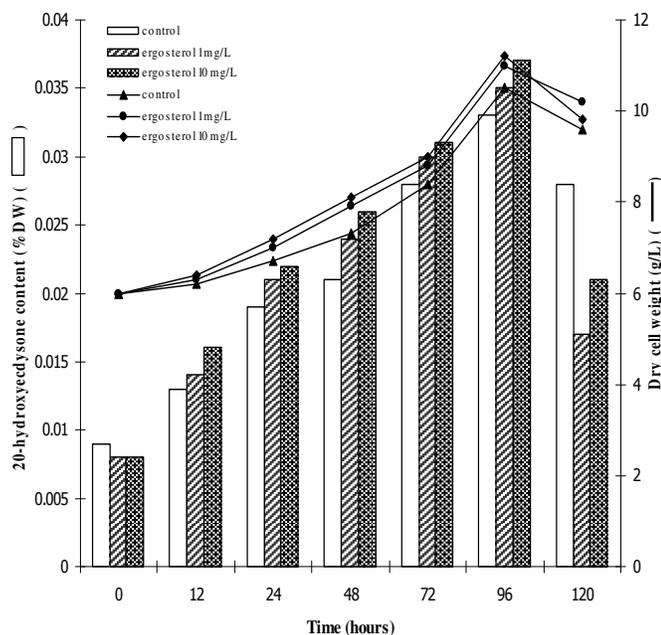


Figure 3. Effect of ergosterol feeding on cell growth and 20-hydroxyecdysone production in *V. glabrata* cell suspension cultures

The maximum amount of 20-hydroxyecdysone was found to be 0.045 %DW after 96 h using 10 mg/L 7-dehydrocholesterol. The increased was about 1.36-fold

over the control cultures. This suggests that improvement of 20-hydroxyecdysone by 7-dehydrocholesterol feeding may be due to its incorporation as a precursor 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 (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, the 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 after 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.

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 in 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 biosynthesis of 20-hydroxyecdysone. These results indicate the possibility of 7-dehydrocholesterol and ergosterol as the natural precursor for ecdysteroids biosynthesis in plant cells. However, there is a need for further studies employing the radiolabeled precursors.

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