CHAPTER V

DISSCUSION

We have demonstrated in this study the existence of the enzyme geranylgeraniol-18-hydroxylase (GGOH-18-hydroxylase) in the leaves of *Croton sublyratus*. The enzyme is presumably involved in the last step of plaunotol biosynthesis in this plant. There has been no report on this enzyme appearing before in the literatures. This study, therefore, provides original information on the biosynthetic pathway of plaunotol and the hydroxylase enzyme discovered from this study can be considered a new enzyme.

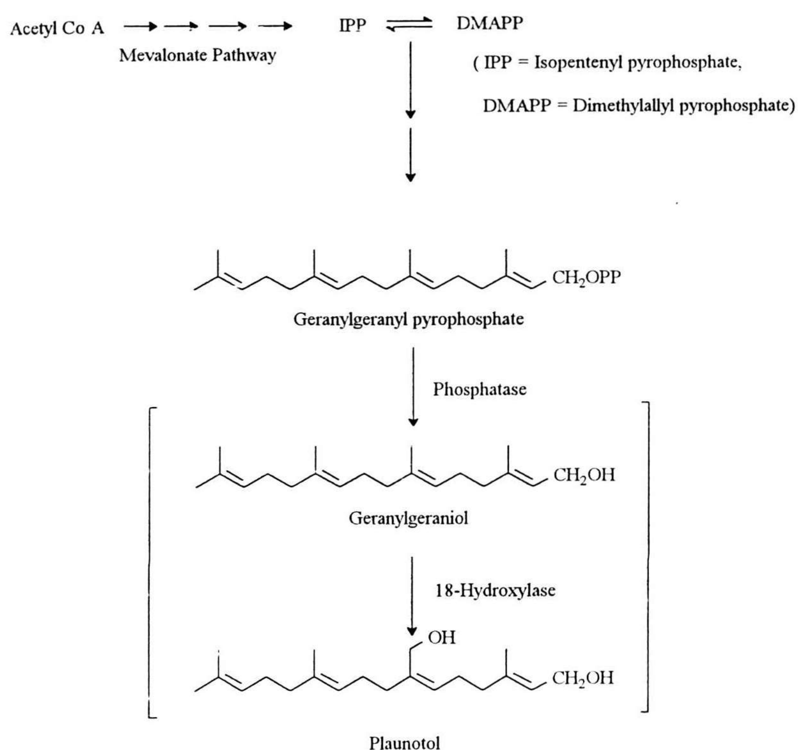
The strategy used in this study in order to obtain information on GGOH-18-hydroxylase enzyme in *C. sublyratus* involved the following steps: 1) detection of the enzyme activity in the leaf cell-free extracts, 2) identification of the enzymatic reaction product 3) enzyme assay and characterization of the partially purified enzyme and 4) electron micrograph observation of the active enzyme function. Disscusion on each of these aspects is given below:

1. Detection of Enzyme Activity in Leaf Cell-Free Extracts

In order to demonstrate the natural existence of GGOH-18-hydroxylase in *C. sublyratus*, it is necessary to be able to detect the enzyme activity in the *in vitro* or cell-free system. This is, however not an easy task since plant enzymes in the cell-free system are usually unstable due to their unfavorable environment and their direct contact with various cellular proteases. Careful optimization of both the buffering pH and chemical composition of an extraction buffer are normally essential to maintain the conformation of an enzyme to be able to exert its activity.

For the detection of GGOH-18-hydroxylase in *C. sublyratus* leaf extracts, a grinding buffer used for enzyme extraction was slightly modified from the grinding buffer used successfully for geraniol-10-hydroxylase extraction from the seedlings of *Catharanthus roseus* (Madyasta, Meehan and Coscia, 1976). This grinding buffur was 100 mM tricine, pH 7.8 containing 5 mM β-mercaptoethanol, 0.4 M sucrose, 10 mM EDTA, 1 mM DTT, 10 mM $MgCl_2$, 10 mg/ml BSA and protease inhibitors (Boehringer Mainheim protease inhibitor cocktail tablets, 1 tablet per 50 ml buffer solution). It can be seen that the buffer contain a number of chemicals for enzyme protection. These include thiol protective products (β-mercaptoethanol and DTT), protease inhibitors (EDTA, inhibitor cocktail and BSA as competitive protease substrate) and polyhydric alcohol (sucrose).

Using this complete grinding buffer, the resulting cell-free extracts showed the presence of two enzyme activities when [1-^{3}H] GGOPP was used as substrate (Figure 9), namely phosphatase and GGOH-18-hydroxylase. The phosphatase activity could be detected in both 20,000 *g* microsomal fraction and 20,000 *g* supernatant whereas the 18-hydroxylase was found essentially in the 20,000 *g* microsomal fraction (Figure 12). Study on time course plaunotol formation by the 20,000 *g* microsomal fraction clearly showed that GGOPP was first hydrolyzed by the phosphatase to form GGOH and the resulting GGOH was then hydroxylated to form plaunotol. Based on these results, it can be concluded that the later steps of plaunotol biosynthesis are as follow:



2. Identification of the Enzymatic Reaction Product

The detection of GGOH-18-hydroxylase activity in the 20,000 *g* microsomal fraction allowed us to investigate the enzyme in more details. However, a crucial point that had to be accomplished before enzyme characterization could be carried out was to unequivocally demonstrate that the product of this enzymatic reaction was plaunotol. This was examined by employing a number of detection method including TLC, UV-spectrometry, GC-MS and IR-spectroscopy. The reaction product showed the similar Rf value on TLC plate to autentic plaunotol (Figure 24). The UV spectra determined at the TLC position of reaction product and authentic plaunotol were similar (Figure 19). When the reaction product was detected by MS using GC-MS compared with authentic product. The reaction product was, again eluated from the GC column with the same as plaunotol (Figure 20). For CI-MS spectroscopy, molecular ion peak is usually deteced at (M+1) but for the case of plaunotol which is an acyclic diterpene, its structure easily looses the molecular of water from the structure. The molecular ion peak of plaunotol ($(M+1)^+$ = 307) was therefore

undetectable. However, both mass spectra showed the peak of $(M+1)^+$-H_2O at 289 and $(M+1)^+$-$2H_2O$ at 271 (Figures 21, 22).

For IR spectra, both authentic plaunotol and enzymatic product showed very similar IR spectra (Figure 23). Both showed absorption bands of O-H strech at 3328 cm^{-1}, C=C bend at 1640 cm^{-1}, C-H bend at 1459 and 1379 cm^{-1} and C-O strech at 1020 cm^{-1}

Base on this evidences, it is clearly indicated that enzymatic reaction product is plaunotol. Its formation of plaunotol is abviously the result of enzymatic reaction. This conclusion comes from the demonstration that the amount of plaunotol produced was related to the amount of microsomal protein (Figure 18) and incubation time (Figures 15, 16). Therefore, these evidences clearly indicate that plaunotol in the reaction mixture is formed by enzymatic activity of GGOH-18-hydroxylase.

3. Enzyme Assay and Characterization of the Partially Purified Enzyme

In this study, the enzyme GGOH-18-hydroxylase was assayed for it's catalytic activity by the technique has been used previously in our laboratory to determine plaunotol content in *C. sublyratus* leaves (Apacha Vongcharoensathit, 1994). It is simple, rapid, acculate and allows many samples to be assayed at the same time. It can detect plaunotol at the amount of as low as 1 nmole and the standard were gives a linearity of area-content relationship up to the level of 15 nmole of plaunotol (Figure 15).

The typical TLC-Chromatogram of sample reaction mixture (Figure 14) shows satisfactory separation of plaunotol from its substrate (GGOH) and other compounds present in the reaction mixture. The assay system is, therefore, highly specific to the detection of plaunotol since only the peak area of plaunotol in the chromatogram is used for determining the formation of plaunotol.

By applying such a method of enzyme assay, the enzyme activity of GGOH-18-hydroxylase in 20,000 *g* microsomal fraction could be characterized. The enzyme activity under the assay conditions was proportional to protein concentration at the range of 20-80 μg/ml (Figure 18). It exhibited high substrate specificity to GGOH (C-20) as compared with its related acyclic farnesol (C-15) or geraniol (C-10).

For cofactor requirement, the hydroxylation activity of the microsomal fraction was found to depend on NADPH which was the best electron donor tested (Table 3). NADH could also substituted for NADPH with the activity about 70 % of NADPH. However, the hydroxylation of geranylgeraniol from microsomal fraction of *C. sublyratus* could also detect without cofactor with the activity about 54 % of NADPH. The reason of these is still not clear.

It has been reported that the hydroxylation in plant requires cofactors as external electron donor (or reducing agent). These cofactors are NADPH and NADH. The mechanism of these two reducing agent are shown in Figure 30. NADH and NADPH are reduced pyridine compounds. Both are two electron-hydride donating agent with a potential of -0.320 volt (Lowe and Ingraham, 1974). The hydride ion is donated readily to produce a stable pyridinium ring. The oxidized form of the compounds are NAD^+ and $NADP^+$ (Lowe and Ingraham, 1974).

All of plant cytochrome P-450 monooxygenase, include hydroxylase, utilized NADPH preferentially as the external electron donor. NADH could be substituted for NADPH as sole electron donor but it was less efficient (West, 1980). Accumulated evidence suggests that these redox proteins act as sources of electrons for monooxygenase reaction. The NADPH-specific flavoprotein, called NADPH-cytochrome P-450 reductase, supply electron to cytochrome P-450 (or hydroxylase enzyme), heme oxygenase, and squalene epoxidase, whereas the NADH-specific flavoprotein, called NADH-cytochrome*b5* reductase, in collaboration with cytochrome *b5*, donates reducing equivalents to fatty acid CoA desaturation (Tanaguchi, Imai and

Sato, 1984). However in the case of *trans*-cinnamic acid-4-hydroxylase from potato and sweet potato, NADH was completely ineffective (West, 1980 quoting Tanaka, *et al.*, 1978, Rich and Lamb, 1977).

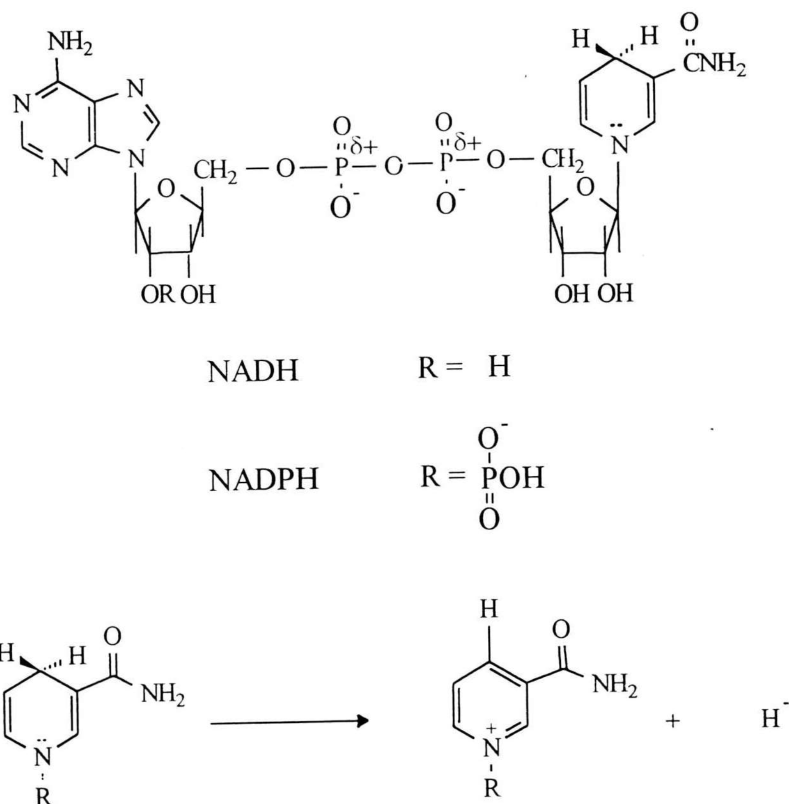


Figure 30 Structure and mechanism of NADH and NADPH

Interesting, the GGOH-18-hydroxylase activity appeared to increase after boiling of 20,000 *g* microsomal fraction for 30 min (Figure 25). The reason behind this observation is still not known. It might be possible that some heat labile inhibitor(s) of the enzyme may be present in the microsomal fraction. Upon boiling, the inhibitors were readily destroyed whereas the hydroxylase enzyme which is presumably composed of very complex structure was more heat-stable. However, the heat stability of the hydroxylase enzyme also showed limitation since after 30 min, its catalytic activity appeared to decrease rapidly (Figure 25).

With respect to pH optimum, the enzyme activity in the heated microsomal fraction showed its optimum pH at 5.0 (Figure 26). This is different from other cytochrome P-450-related hydroxylases which have been reportes in the range 6.8 to 7.8 (Madyasta, Meehan and Coscia, 1976; Higashi,1985; Karp, 1990; Tang and Suga, 1994; Hallahan, 1991)

4. Electron Micrograph Observation of the Active Enzyme Function

The physical structure of microsomal fraction which contained geranylgeraniol-18-hydroxylase activity were observed by negative staining electron microscope (Figure 29). The results showed the presence of particles with diameter from 20 to 60 nm. As expected, the GGOH-18-hydroxylase is a complex enzyme which contain not only the GGOH-18 hydroxlase activity but also other electron transport proteins. Because of time limitation, the purification of geranylgeraniol-18 hydroxylase enzyme was not carried out in this study. To prove this, it requires the reconstitued system such as, NADPH cytochrome P-450 reductase; NADH cytochrom*b5* reductase; cytochrome*b5*, during the incubation of the enzyme.

The electron micrograph of membrane bounded proteins has been studied previously. (Tanaguchi, Imai and Sato, 1984). Negative staining electron micrograph of 4 electron transport proteins include NADPH cytochrome P450 reductase, cytochrome P450, NADH-Cytochrome*b5* reductase and Cytochrome*b5* showed single wall vesicles with similar size distribution.