CHAPTER IV

RESULTS

1. Detection of the Enzyme Activity of Geranylgeraniol-18-Hydroxylase

In this study, the crude enzyme extract prepared from the leaves of *Croton sublyratus* was first incubated with the radioactivelly labelled [1-^{3}H] geranylgeraniol pyrophosphate ([1-^{3}H] GGOPP). The reaction mixture consisted of 83 mM tricine, pH 7.8, 0.045 nM [1-^{3}H] GGOPP (280,000 dpm), 0.8 mM NADPH and 100 µl of the crude enzyme extract, in a total volume of 150 µl. Boiled enzyme was used as a control in this experiment. After 60 min of incubation, the incubation mixtures of both the sample and the boiled control were subjected to TLC and detected for the labelled tritrium compounds by using TLC-radioscanner. Authentic compounds of geranylgeraniol and plaunotol were also run on the same TLC plate but detected by TLC densitometer (λ210 nm). The peak positions of both compounds were used for checking the positions of the reaction products. Figure 11 shows the TLC chromatogram of the standards and the radiochromatograms of the reaction mixtures. It can be seen that in the boiled control (Figure 11 C), there were no conversion of [1-^{3}H] GGOPP to other compounds whereas in the sample mixture(Figure 11 B) there appeared some new radioactivelly labelled products. Amongs these, there were two products showing the same Rf values as reconstruction control of geranylgeraniol (GGOH) and plaunotol.

2. Detection of the Enzyme Activity in Different Centrifugal Pellets of *C. sublyratus* Kurz. Leaves

In order to check roughly which membrane fraction(s) in the crude extract that contained the geranylgeraniol-18-hydroxlase activity, a differential centrifugation was performed. In this study, the pellets of 3,000 *g* and 20,000 *g* were prepared and detected for the presence of the enzyme activity. For comparison, the 20,000 *g* supernatant was also saved and examined. The detection was, again carried out using

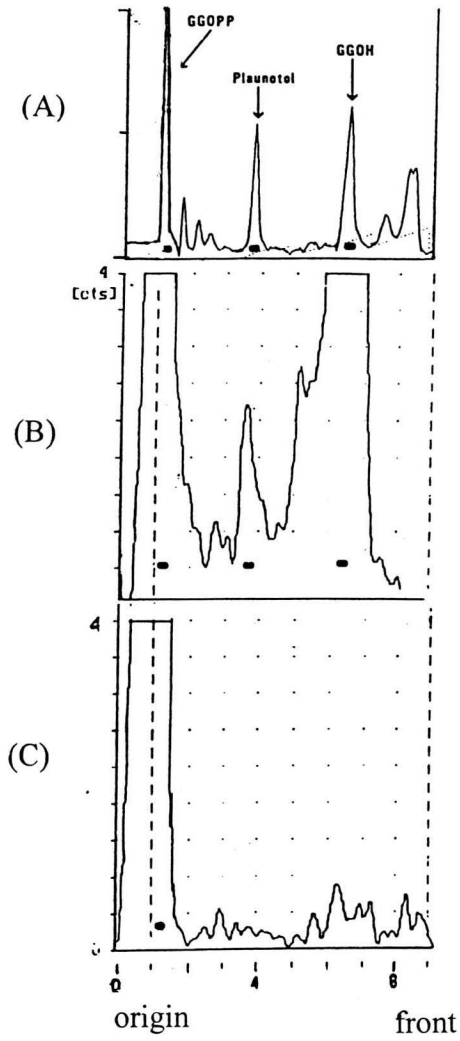


Figure 11 TLC-radiodetection of the enzyme activities converting geranylgeranyl pyro phosphate (GGOPP) to geranylgeraniol (GGOH) and plaunotol by the crude enzyme extract prepared from *Croton sublyratus* leaves.
[1-^{3}H] GGOPP (280,000 dpm) was used as a starting radioactively labelled substrate.

A) TLC-chromatogram showing the positions of GGOPP, GGOH and plaunotol

B) TLC-radiochromatogram of the complete incubation mixture of 60-min incubation

[1-^{3}H] GGOPP as substrate and the resulting TLC-radiochromatograms were analyzed. As shown in Figure 12, the 3,000 *g* pellet showed very low radioactive peaks at the positions of GGOH and plaunotol. The 20,000 *g* pellet, on the other hand, clearly showed radioactive peaks of both compounds in the radiochromatogram. The intensity of the peak at plaunotol position appeared to be more than that of GGOH. For the 20,000 *g* supernatant, only the peak at GGOH position was essentially detected. These results indicated that the geranylgeraniol-18-hydroxylase activity was present in the 20,000 *g* pellet. It was, therefore, used throughout in this study and was called "microsomal fraction".

3. Time-Course of the Conversion of [1-^{3}H] Geranylgeranyl Pyrophosphate to Putative Plaunotol

In order to understand the sequence of the conversion of [1-^{3}H] geranylgeranylpyrophasphate ([1-^{3}H] GGOPP) to other radioactivelly labelled compounds observed in Figures 11 and 12, a time-course of the conversion was studied. In this experiment, [1-^{3}H] GGOPP was incubated with freshly prepared 20,000 *g* microsomal fraction under the same incubation mixture. The mixture was taken at the times 5, 30, 60, 120 and 180 min and extracted with ether before subjected to TLC-radioscan analysis in order to avoid the interference of the high radioactivity [1-^{3}H] GGOPP. As shown in Figure 13, it was found that [1-^{3}H] GGOPP was rapidly converted within 5 min to a major peak corresponding to the position of geranylgeraniol (GGOH) and a few minor peaks with lower Rf values. The peak of GGOH increased maximally at 30 min followed by a continuous decrease of the peak until almost disappearing at 180 min. Simultaneously, the radioactive peak corresponding to the position of plaunotol showed a continuous increase during the whole time-course of the incubation. These results suggested that [1-^{3}H] GGOPP in the incubation mixture was converted to GGOH before being hydroxylated to form putative plaunotol.

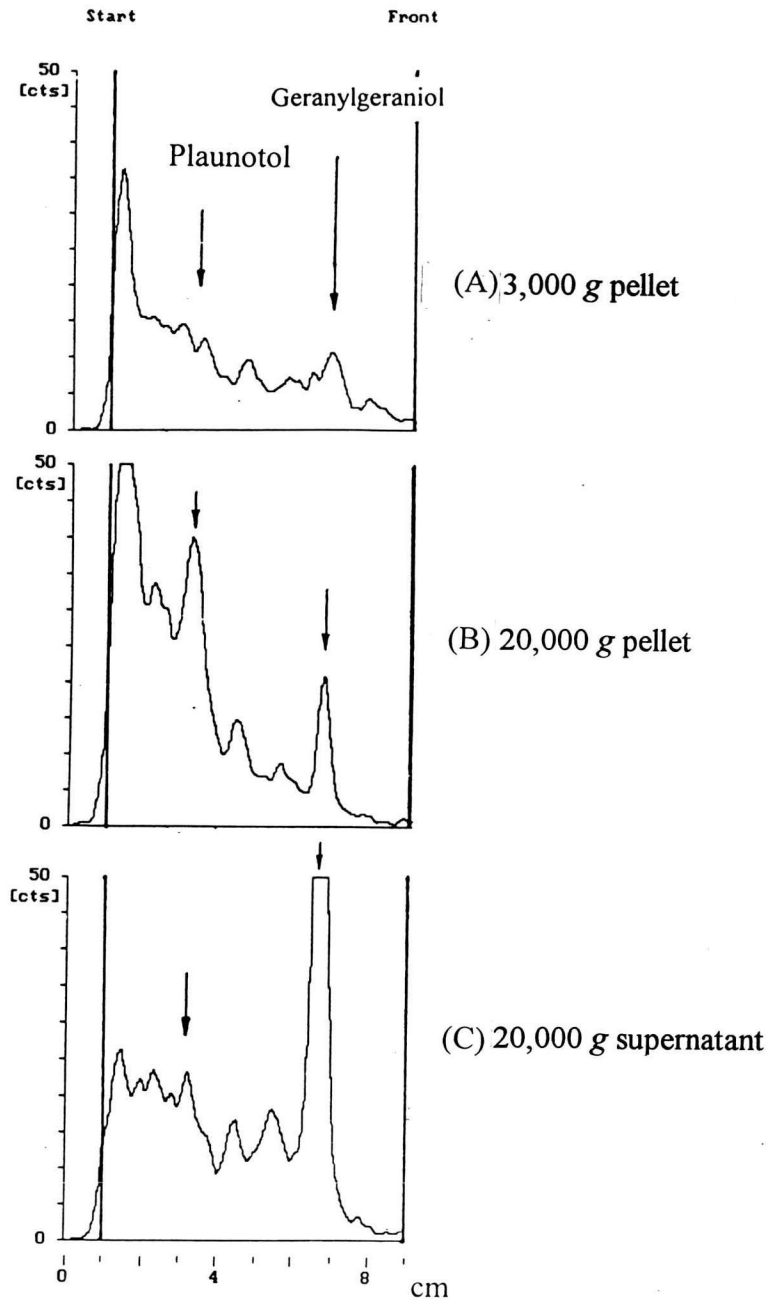


Figure 12 TLC radiochromatograms of the reaction mixtures containing [1-^{3}H] GGOPP and either (A) 3,000 *g* pellet, (B) 20,000 *g* pellet, or (C) 20,000 *g* supernatant. Each enzyme fraction was obtained by a differential centrifugation as described in Materials and Methods, section 3

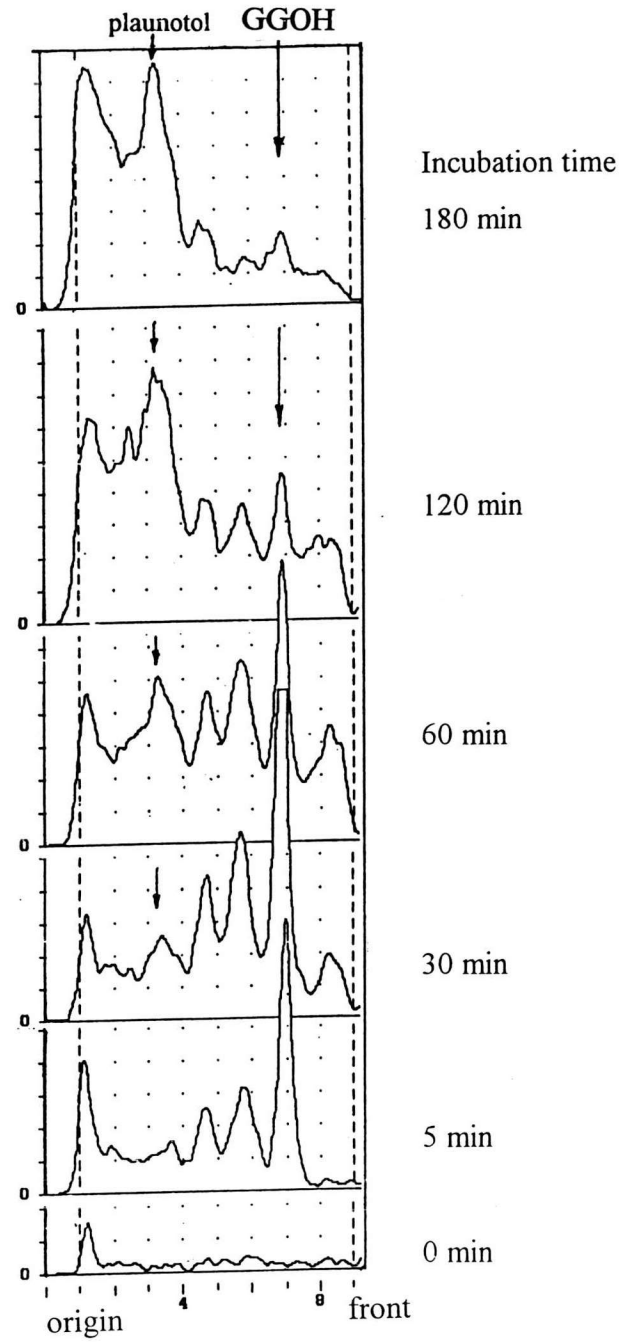


Figure13 Time-course study of the conversion of [1-^{3}H] GGOPP to GGOH and plaunotol by the 20,000 *g* pellet fraction of *Croton sublyratus* leaves

4. Enzyme Assay for Geranylgeraniol-18-Hydroxylase Activity

Since radioactivelly labelled GGOH was not commercially available, the enzyme assay for geranylgeraniol-18-hydroxylase activity had to be carried out using non-labelled substrate. In doing this, the technique of TLC-densitometry described previously was adopted (Apacha Vongcharoensathit, 1994). In pratice, the reaction occuring in the incubation mixture was terminated by ether extraction. The ether extract was concentrated and subjected to TLC-densitometer using the wavelength of 210 nm to produce a chromatogram. The chromatogram was essentially consistent with the visible TLC pattern obtained after exposing with iodine vapour (Figure 14). It showed a relatively clear separation of putative plaunotol peak from other compounds. The substrate, geranylgeraniol, appeared at the Rf 0.81 while the enzymatic product which was on the same position as standard plaunotol appeared at the Rf 0.46. From the chromatogram, the area under the peak of plaunotol was used for estimating the content of newly-formed plaunotol. This was done by the peak area/content conversion using the standard curve of plaunotol (Figure 15) which showed linearity relationship between 0.5 to 15 nmol of plaunotol. The regression analysis and the correlation coefficient was found to be 0.996994 and its linear slope was 1.928.

5. Time-Course of the Conversion of Geranylgeraniol to Putative Plaunotol

To confirm that GGOH was the immediate precursor of plaunotol, a time-course study was carried out to monitor the conversion of GGOH to plaunotol which was catalyzed by the microsomal fraction of *C. sublyratus* leaves. Using the method of enzyme assay described previously, it was found that the content of putative plaunotol was sharply increased in the reaction mixture after 30 min incubation (Figure 16). Thereafter, the reaction proceeded slowly at least for 3 hr as shown in Figure 17.

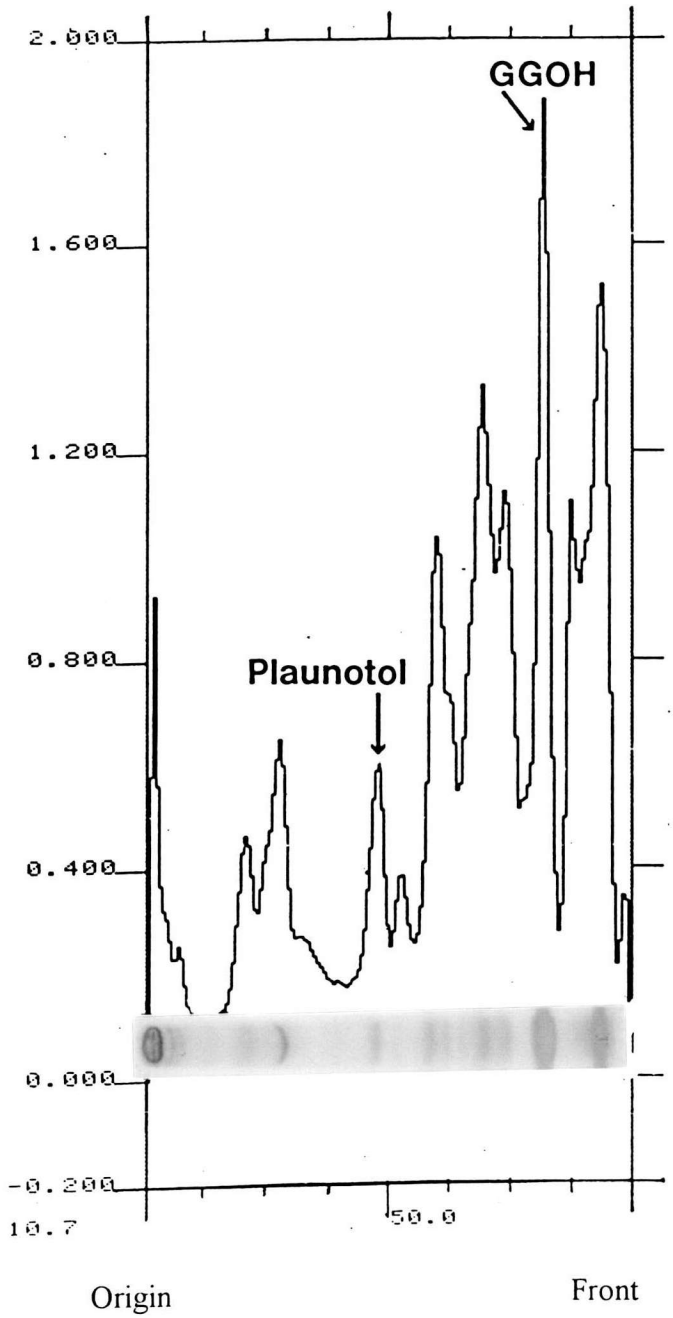


Figure 14 A typical TLC-chromatogram of the reaction mixture separated by TLC and scanned by TLC scanner (λ=210nm). the Rf values of plaunotol and geranylgeraniol are 0.46 and 0.81, respectively.

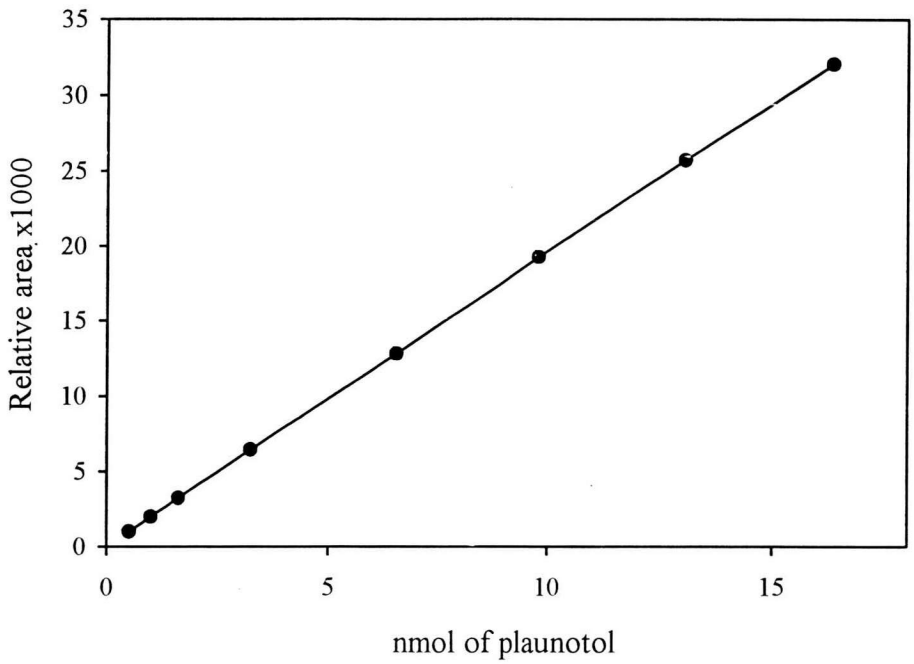


Figure 15 Standard curve of authentic plaunotol

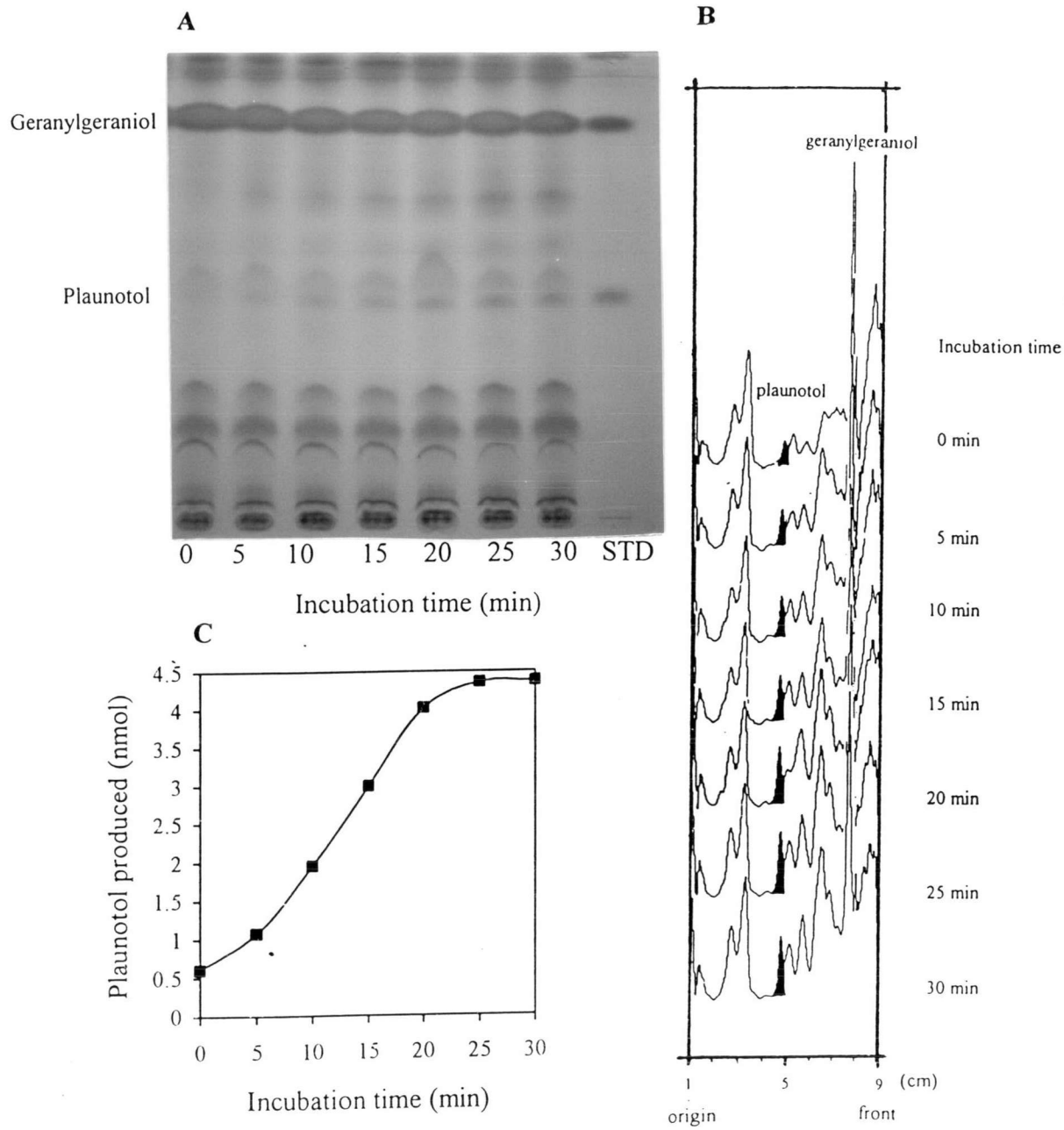


Figure 16 (A) TLC patterns of the reaction mixtures of different incubation times

(B) TLC chromatograms obtained from the scanning on TLC plates shown in (A). The peaks with block color are corresponded to the position of plaunotol.

(C) The graph showed the formation of putative plaunotol during 30 min incubation. The values of plaunotol produced was estimated from its calibration curve.

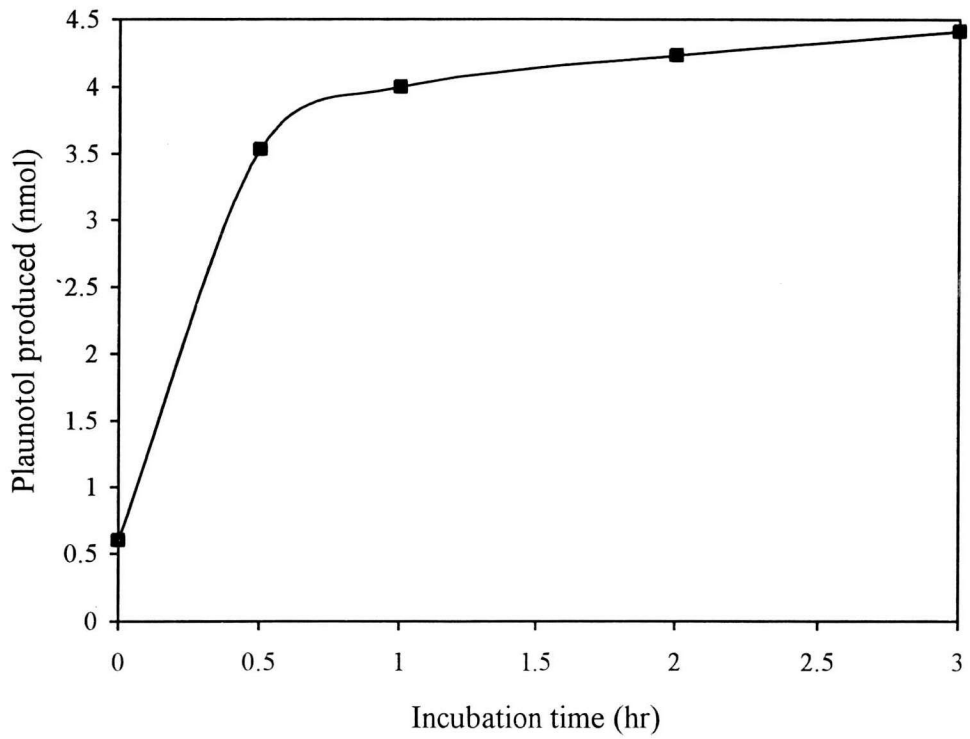


Figure 17 The formation of putative plaunotol during 3-hr incubation. The method of this study was similar to that described in Figure 16

6. Relationship Between the Amount of Microsomal protein and the Enzyme Activity

To ensure that the formation of putative plaunotol was the result of enzymatic reaction, a relationship between the amount of microsomal protein and enzyme activity was examined. In this study, various amounts of microsomal protein ranging from 10 to 170 μg protein/ml were added in the tubes of the incubation mixture and incubated for 30 min. It was found that the amount of putative plaunotol formed in the reaction mixture was dependent on the amount of microsomal protein, especially between the protein concentration range of 20-80 μg/ml (Figure 18). This confirmed that the putative plaunotol was formed by enzymatic reaction.

7. Identification of the Enzymatic Reaction Product

Identification of the enzymatic reaction product was carried out with large-scale (250-fold) incubation. The reaction product purified by preparative TLC was elucidated for its structure by 3 different methods, namely UV-spectroscopy, GC-MS (CI) and IR-spectroscopy.

For UV-spectroscopy, the oilly enzymatic product showed a UV-spectrum similar to the authentic plaunotol (Figure 19). For GC-MS, it was eluted from a GC capillary column (9.25 min) with the retention time close to the standard plaunotol (9.31 min) (Figure 20). The CIMS spectra of both the product and authentic plaunotol were also very similar(Figure 21). Both mass spectra showed the peaks of $[(M+1)^+-H_2O]$ at 289 and $[(M+1)^+-2H_2O]$ at 271.(Figure 22). Moreover, the IR spectra of the product and plaunotol were also similar (Figure 23). Both showed characteristic absorption at 3350, 2923, 1645, 1379 and 1016 cm^{-1}. The results, therefore, confirmed that the enzymatic product was plaunotol.

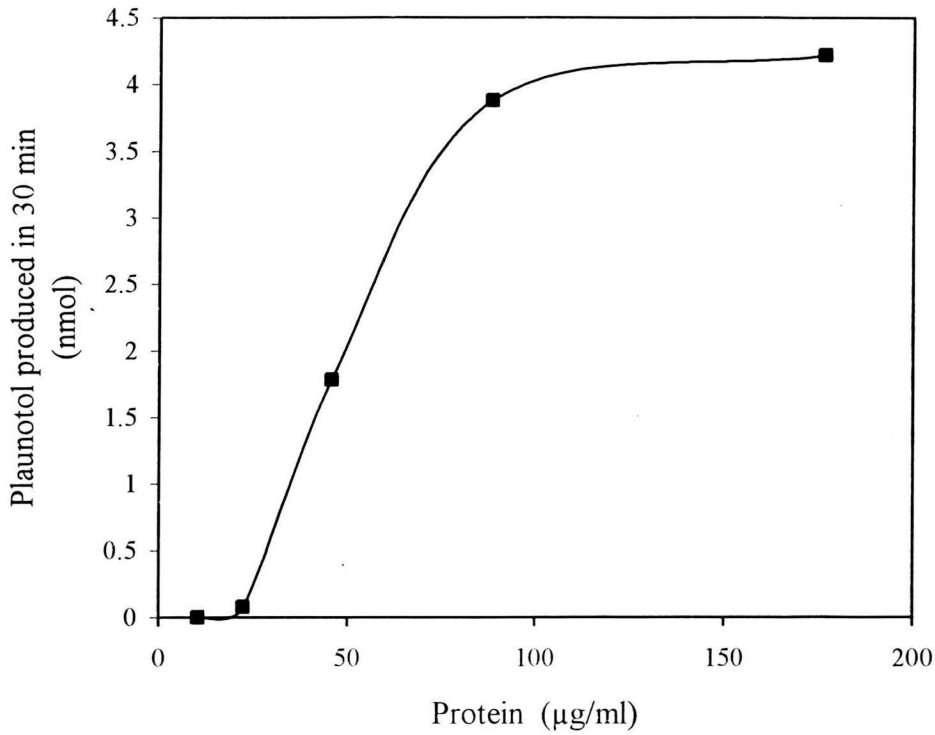


Figure 18 The effect of the amount of microsomal protein on the conversion of geranylgeraniol to plaunotol

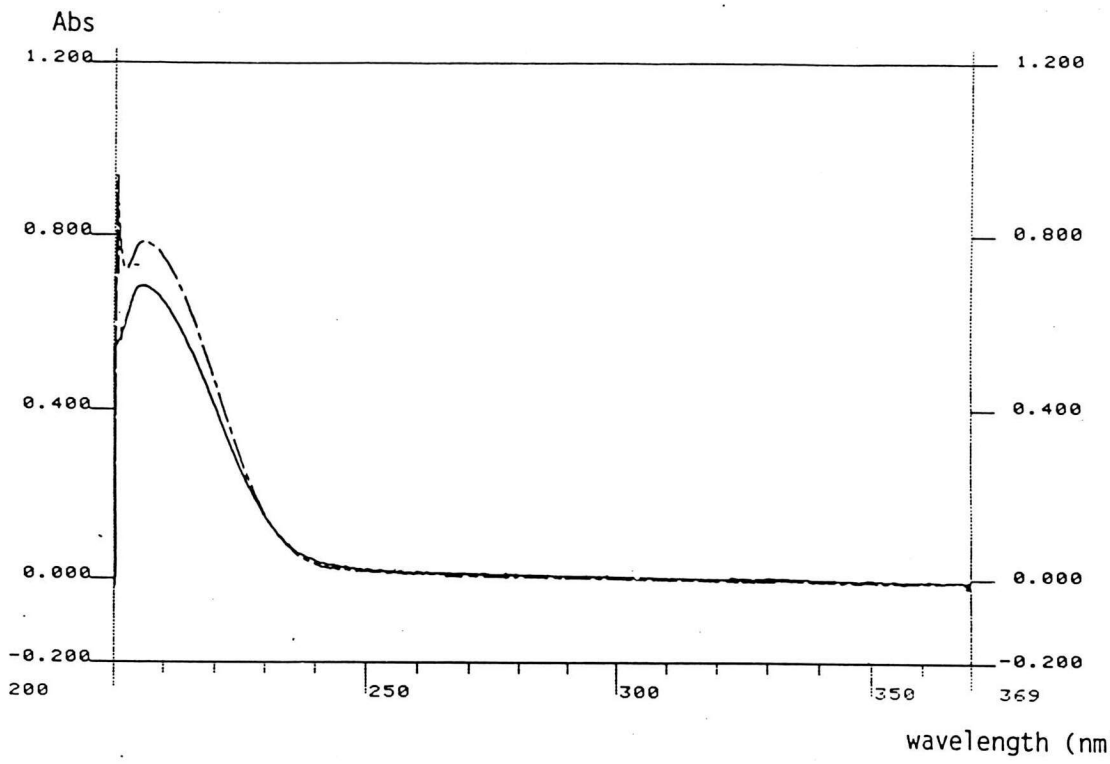


Figure 19 UV-absorption spectra of standard plaunotol (— - — - —) and enzymatic product (— — — — —)

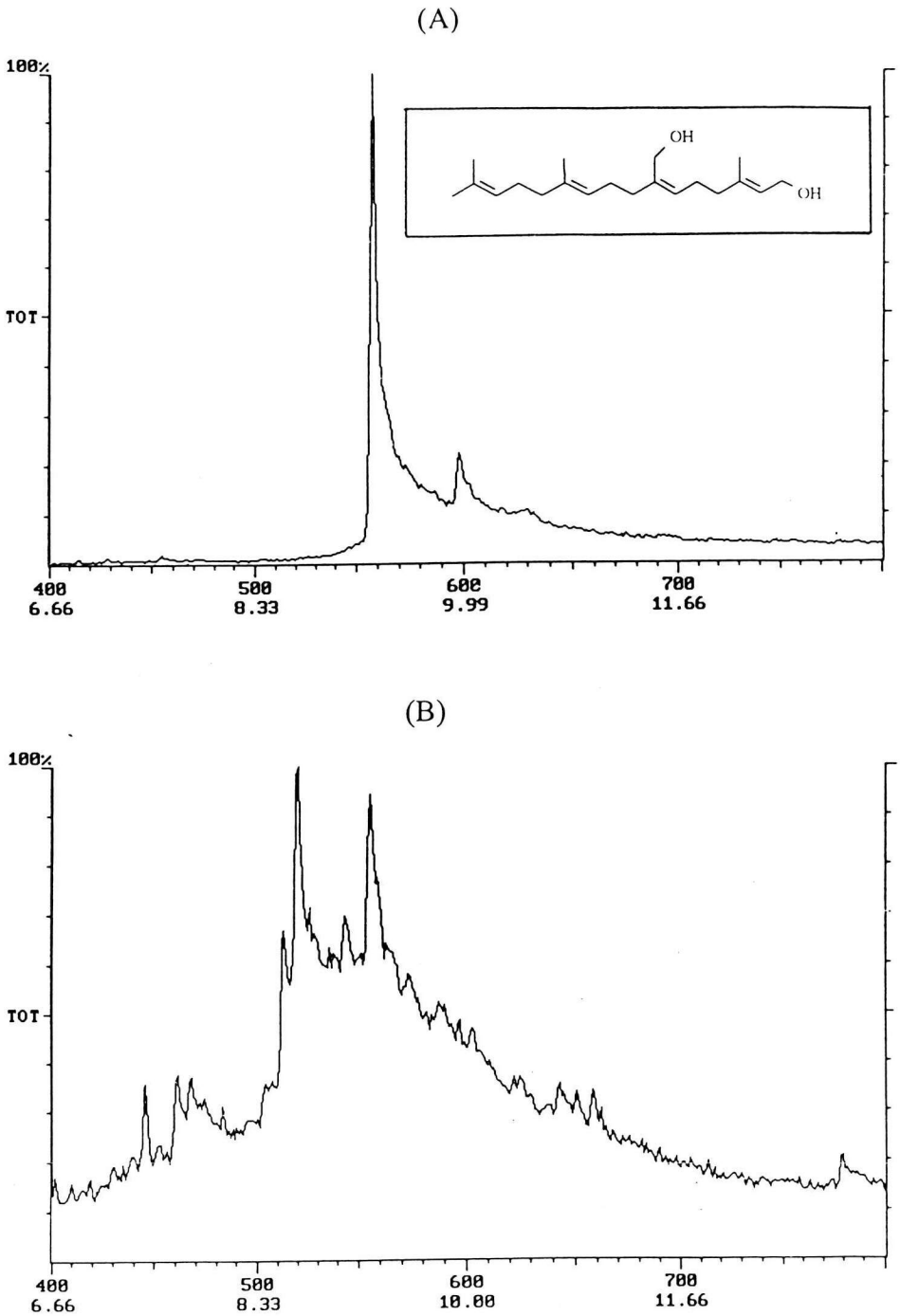
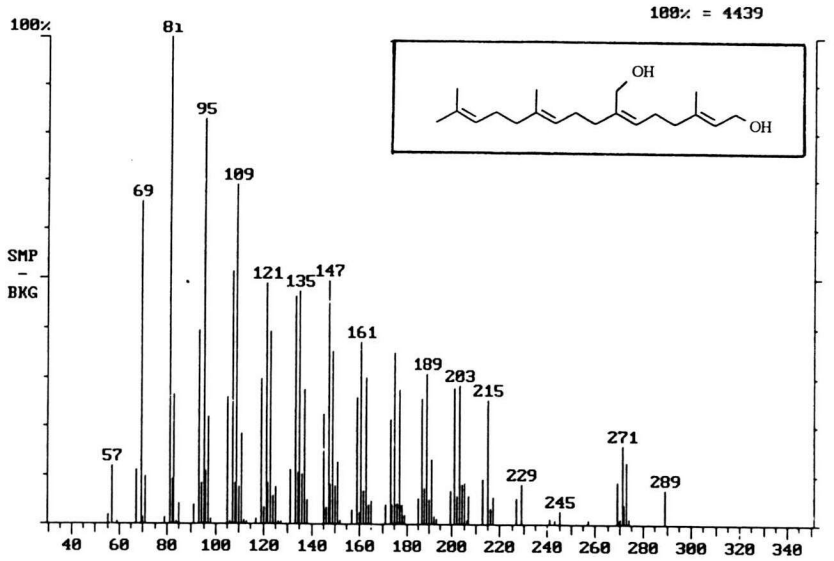


Figure 20 GC chromatogram of standard plaunotol (A) and the isolated product from enzymatic reaction (B).

(A)



(B)

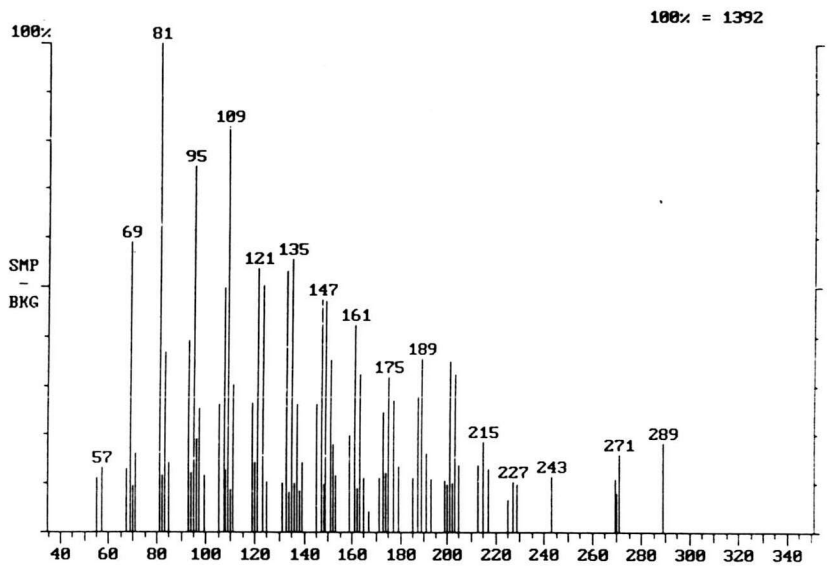


Figure 21 CIMS of standard plaunotol (A) and the isolated product from enzymatic reaction (B).

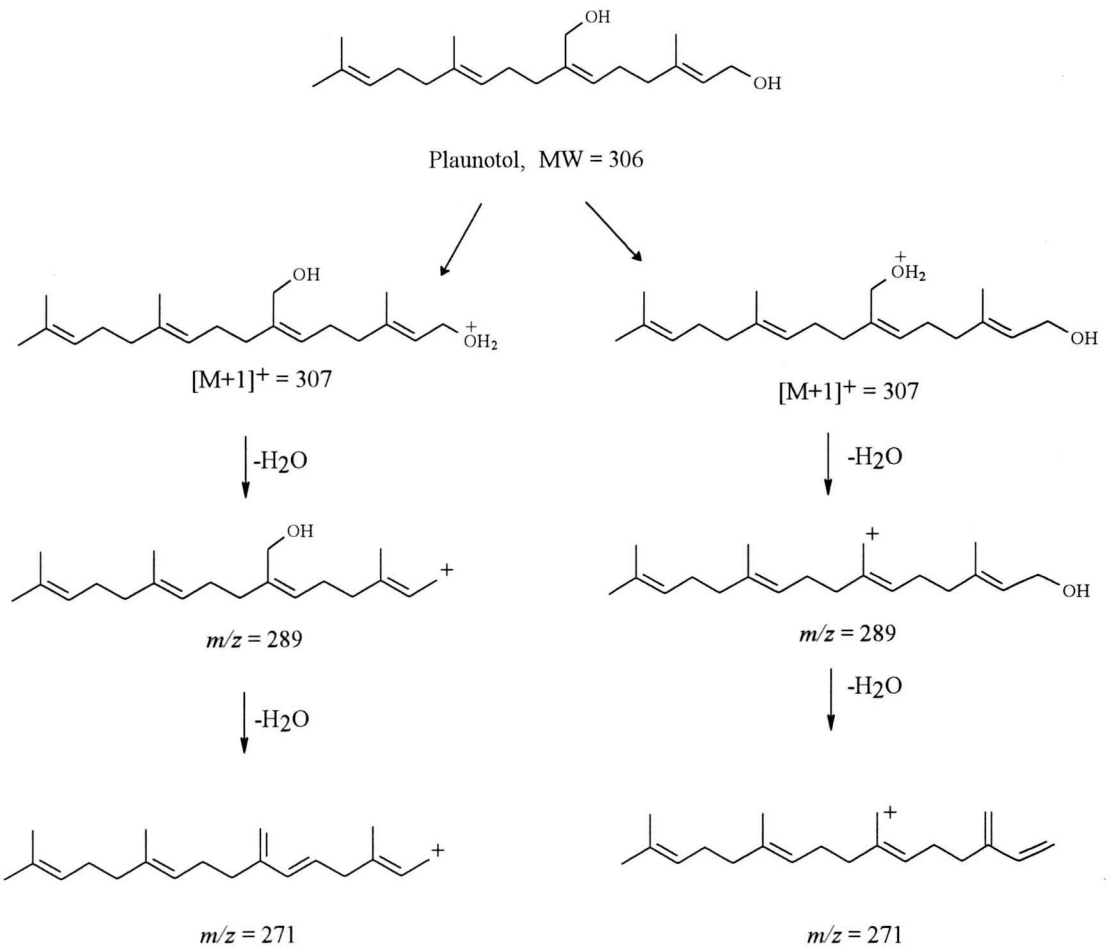


Figure 22 CI mass fragmentation of plaunotol

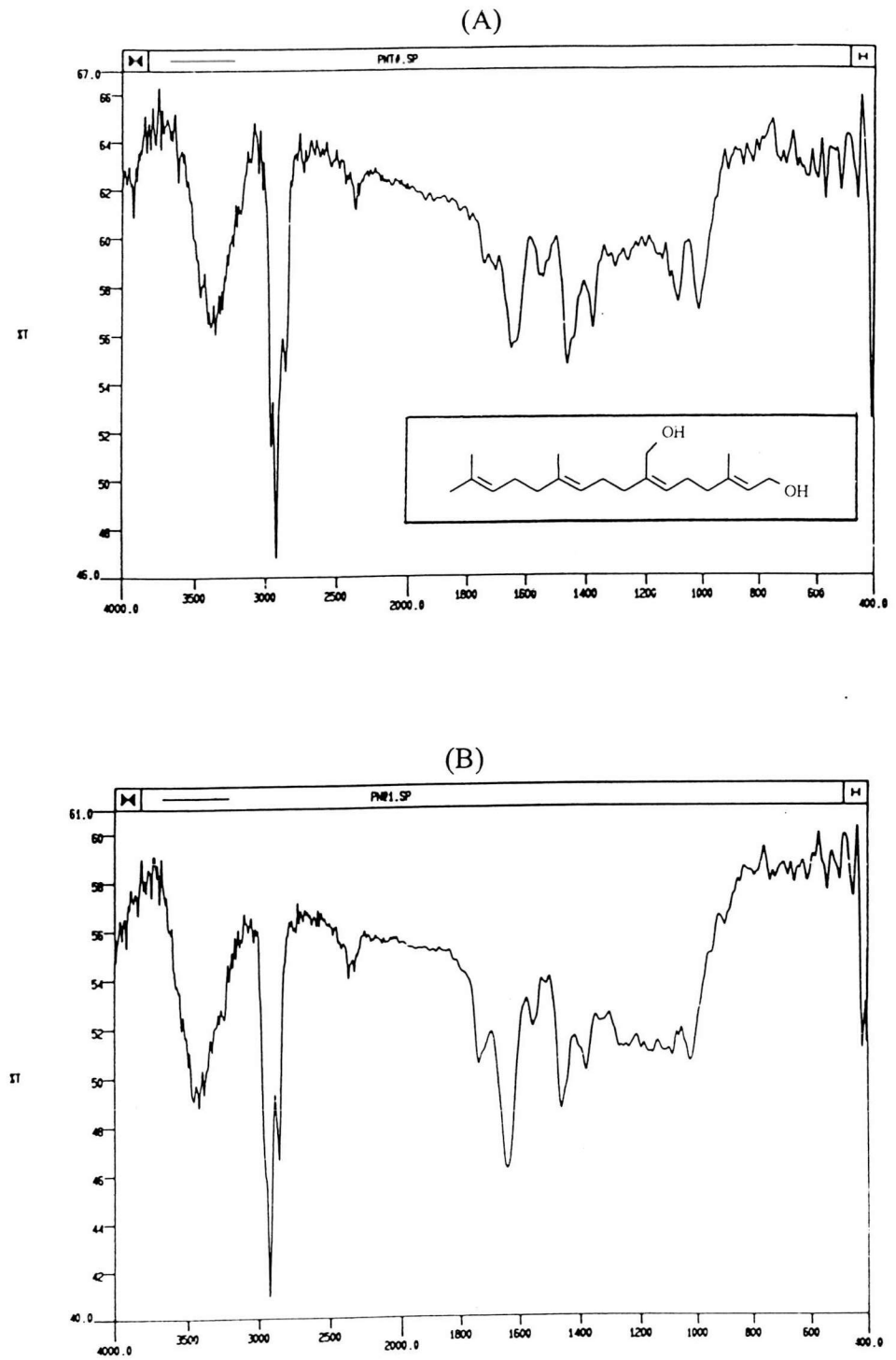


Figure 23 IR spectra of standard plaunotol (A) and the isolated product from enzymatic reaction (B).

8. Effect of Microsome Boiling on the Enzyme Activity

This experiment was carried out based on the observation that the boiled control frequently showed higher enzyme activity than the normal sample (Figure 24). To confirm the positive effect of microsomal boiling on the enhancing enzyme activity, a time-course of the boiling microsome was investigated. In this experiment, the microsomal fraction was boiled at 100 °C for 5, 10, 20, 30, 45 and 60 min before being put into the reaction mixture. After 30 min, each mixture was extracted with ether and determined for plaunotol content by the same standard enzyme assay. As shown in Figure 25, the microsomal fraction showed an increase in the enzyme activity during boiled and reached the maximum at 30 min. Thereafter, the enzyme activity declined rapidly and was almost nondetectable after 60 min of boiling. Therefore, the results clearly showed that boiling of the microsomal fraction did increase the activity of the hydroxylase enzyme.

9. Effect of pH on the Enzyme Activity

The normal pH used in the standard reaction mixture was 7.8. However, an enzyme usually exhibits a pH optimum in catalyzing a reaction. This experiment, therefore, examined the rate of plaunotol formation at different pH. It was found that the enzyme had its pH optimum in a narrow range around pH 5 (Figure 26). Above or below this value, the enzyme activity decreased sharply.

10. Effect of Cofactors on the Enzyme Activity

Incubation of the reaction mixture in the presence of either NADPH or NADH showed that NADH could also replace NADPH but with about 70% of the enzyme activity (Figure 27 and Table 3). However, the formation of plaunotol could also be detected in the absence of the cofactor. The activity was about 54% of the complete reaction mixture.

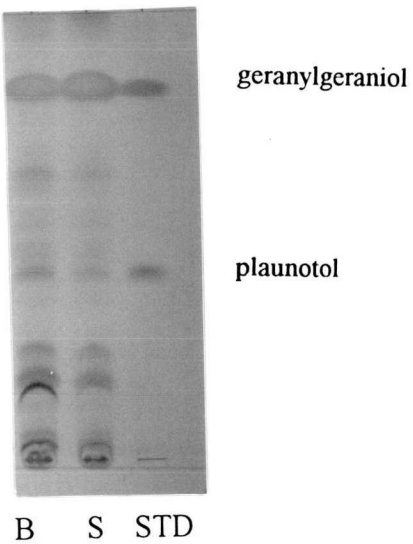


Figure 24 Comparison between the boiled (B) and unboiled (S) microsomal fractions on the conversion of geranylgeraniol to plaunotol

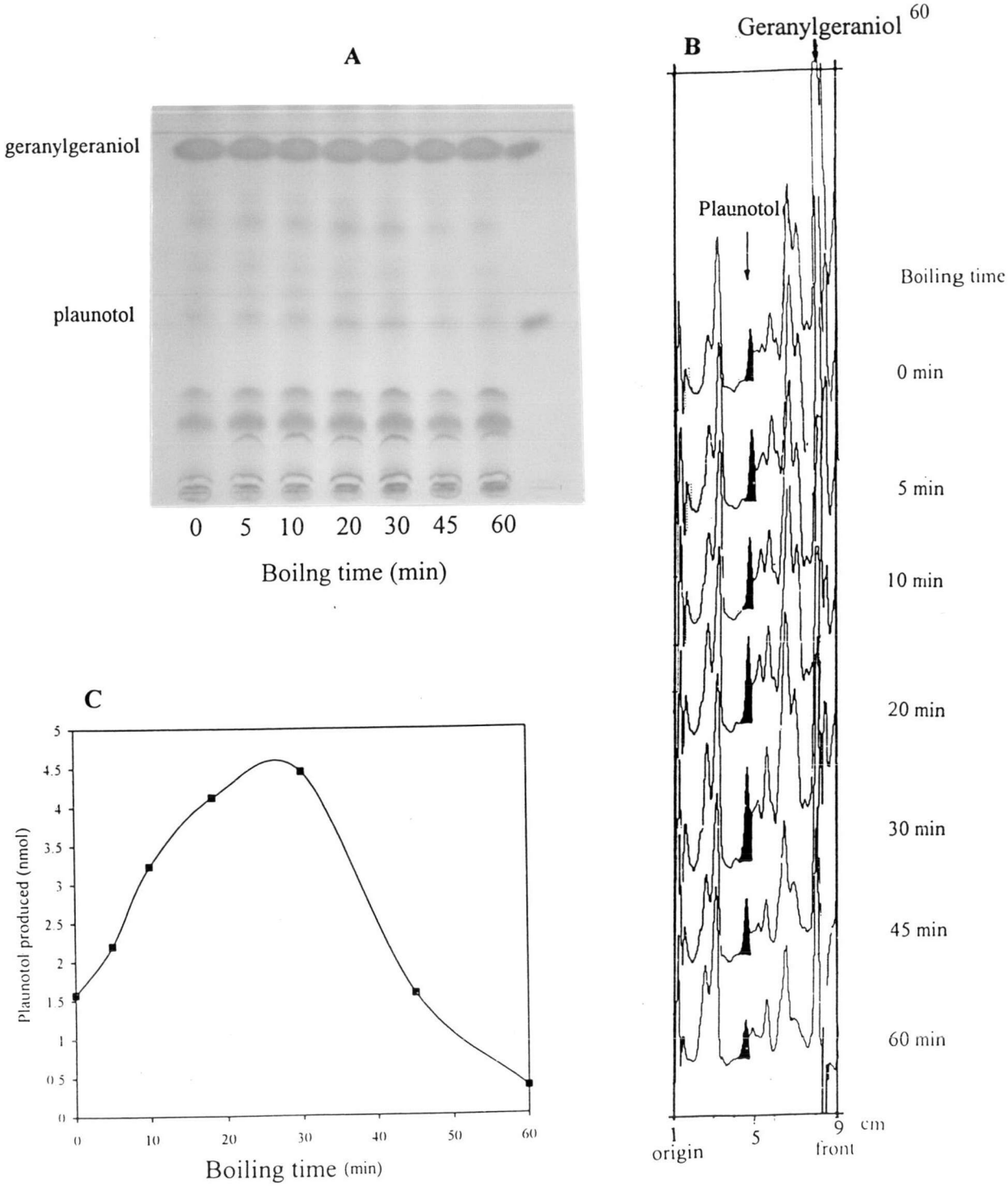


Figure 25 (A) TLC patterns of the reaction mixtures of different boiling times

(B) TLC chromatograms obtained from the scanning on TLC plates shown in (A). The peaks with block color are corresponded to the position of plaunotol.

(C) The graph showed the formation of putative plaunotol from different boiling time incubation. The values of plaunotol produced was estimated from its calibration curve.

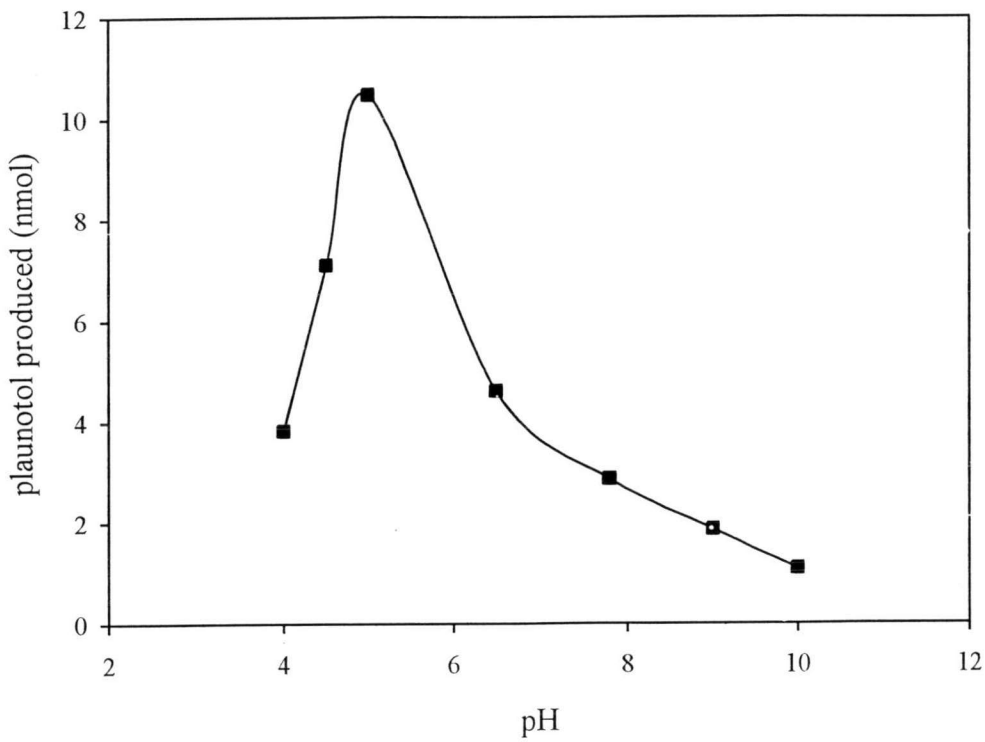


Figure 26 Effect of pH on the 20,000 *g* microsomal activity in the conversion of geranylgeraniol to plaunotol

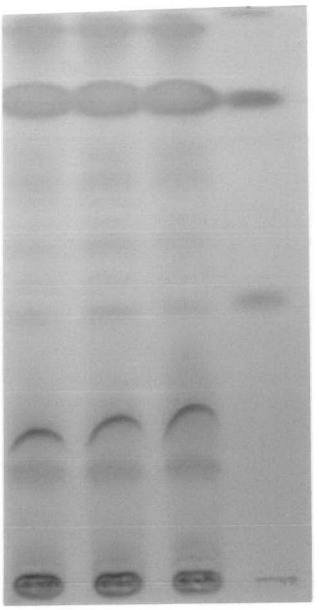


Figure 27 Effect of cofactor on microsomal activity

1) = + NADH

2) = + NADPH

3) = Blank (No cofactor)

4) = Reconstruction control (plaunotol and geranylgeraniol)

Table 3 Effect of cofactor on Enzyme Activity

Experiment	% relative activity*
Control (No cofactor)	54.1
+NADPH	100.0
+NADH	70.4

* 100% relative activity represent 8.33 nmole of plaunotol in 30 min

11. The Enzyme Specificity towards Shorter-Length Substrates

The substrate specificity of the boiled microsomal fraction is shown in Figure 28. It can be seen that only geranylgeraniol (GGOH) could be utilized by the enzyme. Essentially neither farnesol nor geraniol was found to convertto its hydroxy derivatives by the 20,000 *g* microsomal fraction.

12. Electron Micrograph of the Microsome-Containing Geranylgeraniol-18-Hydroxylase Activity

In order to understand the physical structure of the microsomal fraction which contained geranylgeraniol-18-hydroxylase activity, an observation by electron microscope was performed. In this study, the 20,000 *g* microsomal fraction was first boiled for 30 min to precipitate some labile protein and an aliquot was taken for the enzyme assay and found total enzyme activity was 494 nmole/30min/10ml. After being centrifuged at 20,000 *g*, for 20 min the supernatant and the pellet were assayed for the enzyme activity (Table 4) and found that most of the activity was in the supernatant fraction (289 nmole/30min/10min) as compare with the pellet fraction (76 nmole /30 min/10ml). The supernatant was then concentrated by using centricon-10 concentrater. The resulting supernatant was then used for specimen preparation for electron microscope observation. The resulted electron micrograph showed the presence of homogeneous particles with diameters ranging from 20 to 60 nm (Figure 29).

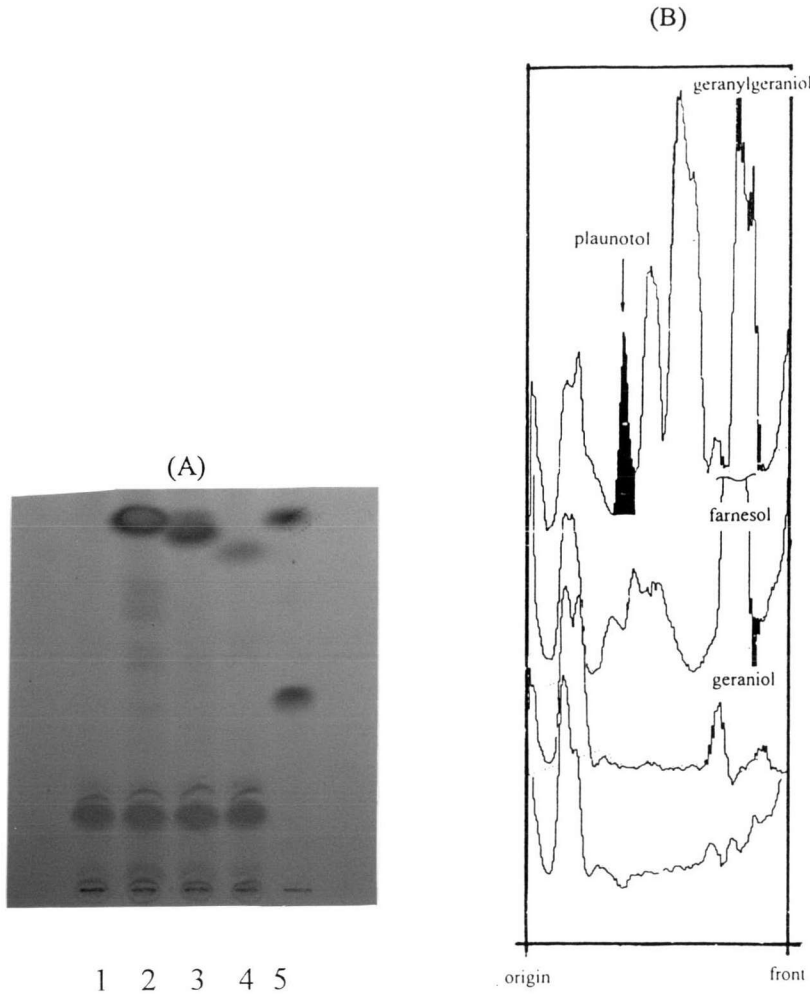


Figure 28 (A) TLC Patterns and (B) TLC chromatograms of the reaction mixture containing shorter-length substrates

1) = Control (no substrate) 2) = Substrate was geranylgeraniol.

3) = Substrate was farnesol. 4) = Substrate was geraniol.

5) = Recostruction control (plaunotol and geranylgeraniol)

Table 4 Enzyme activity and protein content in each fraction

	Total enzyme activity (nmole/30min/10 ml)	Total protein (mg/10ml)
Boiled microsomal enzyme	494	3.4
20,000*g* Pellet from boiled microsomal enzyme	76	2.3
Supernatant from boiled microsomal enzyme	289	1.9

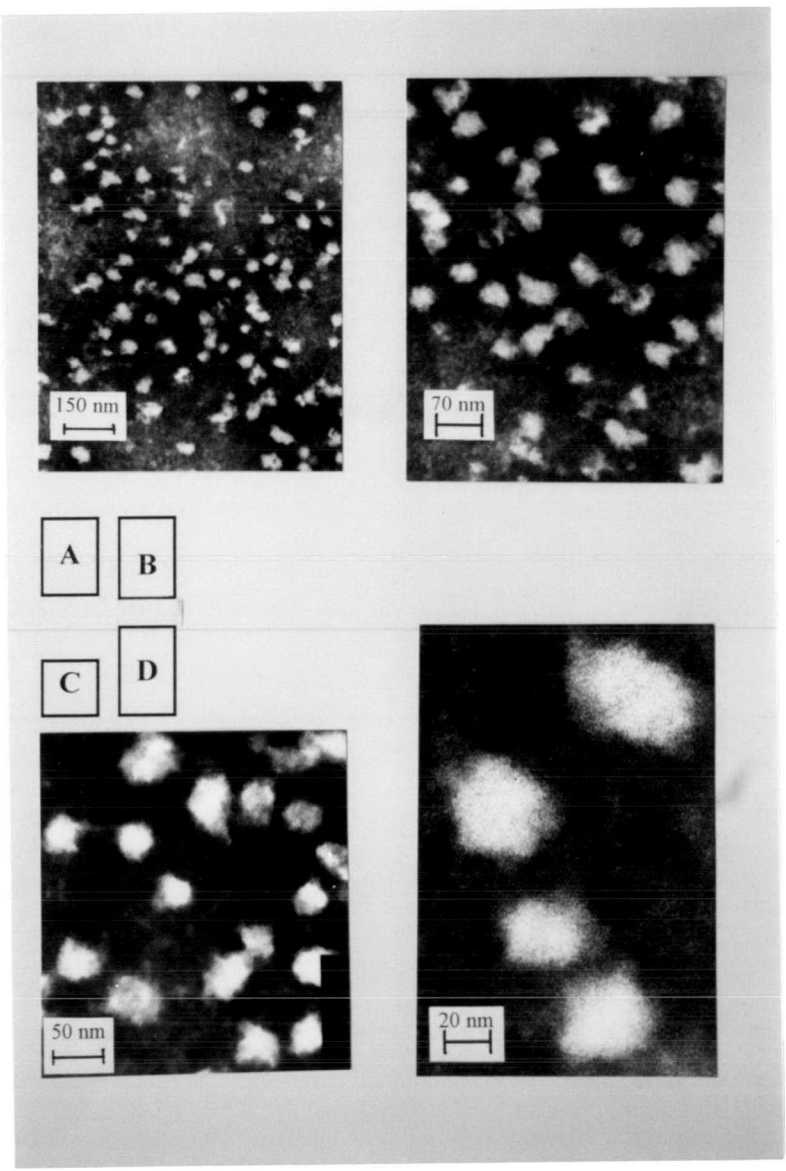


Figure 29 Electron micrographs of the boiled microsomal fraction showing the presence of particles with the diameters ranging from 20 to 60 nm in various magnifications

A = X 75,000 B = X 150,000

C = X 225,000 D = X 495,000