CHAPTER VI
CONCLUSION

The conditions for high amount of enzymatic products of [1-^{3}H]GGMP and [1-^{3}H]GGDP were successfully optimized. By using microsomal proteins from *Nicotiana tabacum* cell suspension culture, [1-^{3}H]GGOH with phosphate donor of CTP was converted to 95% [1-^{3}H]GGMP in Tris/HCl buffer pH 7.0, and was converted to 60% [1-^{3}H]GGDP in MOPS buffer pH 6.5.

The simple purification methods of [1-^{3}H]GGMP and [1-^{3}H]GGDP from reaction mixture was developed into a single step by using MCI gel CHP20P column chromatography.

Feeding experiment of [1-^{3}H]GGOH into leaves of *C. stellatopilosus* exhibited radiolabeled plaunotol. This confirmed the proposed biosynthesis pathway of plaunotol to be downstream from GGOH. Feeding of [1-^{3}H]GGOH into callus gave unclear radiolabeled plaunotol because there was too less amount of plaunotol occurred. So it could not be further investigated. However, no radiolabeled plaunotol could be detected from feeding of [1-^{3}H]GGOH into cell suspension culture.

Since the previous reports showed that plaunotol formation and its accumulation was found in chloroplast, and GGDP phosphatase activity could be detected both in chloroplast extraction and in cell-free extract of 20,000 x *g* pellet from leaves. Therefore, the purification and characterization of GGDP phosphatase was performed in this fraction of 20,000 x *g* pellet containing chloroplast. GGDP phosphatase was a membrane-bound enzyme that could be removed from a 20,000 x *g* microsomal fraction by 0.1% Triton-X without significant loss of the enzyme activity. The solubilized GGDP phosphatase were separated into two activity peaks, PI and PII, by BioGel A gel fitration chromatography. PI and PII were both partially purified and characterized. PI appeared to be a tetrameric enzyme with the whole *Mr* of 232 kDa and the subunit *Mr* of 58 kDa whereas PII was a monomeric enzyme with the size of 30-34 kDa. The pH optimum was found to be 6.0-6.5 for PI and 6.5-7.0 for PII. PI showed high substrate specificity toward GGDP whereas PII could utilized all GGDP, FDP and GDP (relative activity 100:23:10). For enzyme kinetic values, PI exhibited the value of *K*m 0.2 mM, and *V*max of 278 pkat/mg; and PII exhibit the *K*m value of 0.1 mM, and *V*max of 7530 pkat/mg. For the effect of metal ions (at 1 mM),

PI was inhibited by Mo^{2+} and stimulated slightly by Mg^{2+} whereas PII was inhibited by Mo^{2+}, Mn^{2+} and Zn^{2+} but showed no significant effect by Mg^{2+}. The properties of PI and PII were summarized in table .

Table 16 Properties of PI and PII of *C. stellatopilosus*

Enzyme property	PI	PII
Mr		
native (kDa)	232	34
subunit (kDa)	58	30
Optimum pH	6.0-6.5	6.5-7.0
*K*m (mM)	0.1	0.2
Vmax (pkat/mg)	278	7530
GOH, FOH, GGOH effect	No	No
Substrate specificity	GGPP	GGPP, FPP, GPP
Substrate (GGPP) inhibitor	No	Yes
Ion inhibition	Na_2MoO_4	Na_2MoO_4, $ZnSO_4$, $MnSO_4$, $MnCl_2$, $CoCl_2$

Based on molecular weight and subunits, PI was found to be similar with ADP- glucose pyrophosphorylase which is enzyme found in chloroplast and plays a role in sugar biosynthesis. Meanwhile, the molecular weight of PII was closed to that of the recombinant protein of prenyl diphosphate phosphatase from *C. stellatopilosus* as shown in this study.

Since there was no reports of amino acid sequences of GGDP phosphatase available in database and the partial purification of GGDPase from *C. stellatopilosus* could not be obtained amino acid sequence, the degenerate primers were designed for gene cloning of prenyl diphosphate phosphatase by the consensus between amino acid sequence of prenyl diphosphate phosphatase from database.

The full-length gene of *C. stellatopilosus* encoding for prenyl diphosphate phosphatase showed 2 possible open reading frames, namely ORF1 and ORF2. The ORF1 encoded a protein of 296 amino acids with 888 nucleotides long and the calculated molecular mass was 33.6 kD. And the ORF2 encoded a protein of 266 amino acids with 798 nucleotides long and the calculated molecular mass was 29.9 kD.

Amino acid sequences alignment of prenyl diphosphate phosphatase from *C. stellatopilosus* revealed high homologies with prenyl diphosphate phosphatase, phosphatidic acid phosphatase, and diacylglycerol pyrophosphate phosphatase from other plants. Three areas of conserved sequences of phosphatase motifs were determined. Talon resin is an immobilized metal affinity chromatography (IMAC) using Co^{2+} which can bind to his-tag protein. The ChloroP program predicted no position of chloroplast transit peptide (cTP) of both ORF1 and ORF2 nucleotide sequence, so it cannot be concluded that the location of this enzyme was in chloroplast. The 5-clones of ORF1, ORF2, their truncate forms, and the clone of the amino acid sequences downstream to RR motif were expressed in *E. coli* BL21 CodonPlus (DE3)-RIL strain. The His-tagged protein was obtained in low amount which could be detected by Western Blot techniques. All of 5 recombinant protein of prenyl diphosphate phosphatase from *C. stellatopilosus* exhibited phosphatase activities when using [1-^{3}H]GGDP as a substrate using empty vector as a control. GGOH was an enzymatic product from ORF1, ORF2 and their truncate form. Only ORF1 produced the GGOH as a major product, whereas the others mainly produced GGMP.

The dephosphorylation step of GGPP forming GGOH was determined by various sources of GGDP phosphatase from *C. stellatopilosus* included chloroplast extract from fresh leaves, the partial purified enzyme from Superose 6 gel filtration column for PI and the purified enzyme from UNO Q anion-exchange column for PII, and finally the recombination prenyl diphosphate phosphatases of *C. stellatopilosus*. The results showed that GGMP was an intermediate product during dephosphorylation of GGDP. So we proposed the dephosphorylation steps of GGDP by GGDP phosphatase could be GGDP → GGMP → GGOH.