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Membrane-bound geranylgeranyl diphosphate phosphatases: Purification and characterization from *Croton stellatopilosus* leaves

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Dedicated to Prof. Dr. Rodney Croteau on occasion of his 60th birthday.

Abstract

Geranylgeranyl diphosphate phosphatase is an enzyme catalyzing the dephosphorylation of geranylgeranyl diphosphate (GGPP) to form geranylgeraniol (GGOH). The enzyme activity of GGPP phosphatase was detected in leaves of *Croton stellatopilosus*, a Thai medicinal plant containing plaunotol, a commercial anti-peptic acyclic diterpenoid. Enzymological studies of GGPP phosphatase in *C. stellatopilosis* leaves revealed that the enzyme is a membrane-bound protein that could be removed from 20,000*g* pellet by 0.1% Triton X-100 without significant loss of enzyme activity. The solubilized enzyme preparation was separated into two activity peaks, PI and PII, by BioGel A gel filtration chromatography. PI and PII were both partially purified and characterized. PI appeared to be a tetrameric enzyme with its native molecular mass of 232 kDa and subunit size of 58 kDa, whereas PII was a monomeric enzyme with a molecular mass of 30–34 kDa. Both phosphatases utilized GGPP as the preferred substrate over farnesyl and geranyl diphosphates. The apparent K_m values for GGPP of PI and PII appeared to be 0.2 and 0.1 mM, respectively. Both activities were Mg^{2+} independent and exhibited slightly acidic pH optima, 6.0–6.5 for PI and 6.5–7.0 for PII. The catalytic activities of PII was strongly inhibited by 1.0 mM of Zn^{2+}, Mn^{2+} and Co^{2+}, whereas that of PI was not affected. Both enzyme preparations were very stable upon storage at −20 °C for 45 days without significant loss of phosphatase activity. The presence of GGPP phosphatase enzymes in *C. stellatopilosus* is consistent with its putative involvement in the biosynthetic pathway of plaunotol although whether PI or PII is the actual enzyme involved in the pathway remains to be clarified.

Keywords: *Croton stellatopilosus*; Euphorbiaceae; Geranylgeraniol; Geranylgeranyl diphosphate; Geranylgeranyl diphosphate phosphatase; Acyclic diterpene biosynthesis; Enzyme purification; Enzyme characterization

1. Introduction

Plaunotol (**3**), an acyclic diterpene alcohol with anti-peptic ulcer activity, is an 18-hydroxy derivative of geranylgeraniol (**2**) (GGOH) (Fig. 1). It is one of the simplest natural diterpenoid derivatives which has, so far, been found to be present only in *Croton stellatopilosus* Ohba (Euphorbiaceae) (Ogiso et al., 1978). This plant species, known previously as *Croton sublyratus* Kurz (Esser and Chayamarit, 2001), is a Thai medicinal plant called locally as ''Plau-Noi''. It has been used traditionally as stomachic, anthelmintic and dermatologic agent (Ponglux et al., 1987). Its leaves, containing 0.3–0.5% dry weight of plaunotol (**3**) (Vongchareonsathit and De-Eknamkul, 1998), have been used as a raw material for extraction and purification of

Abbreviations: GGPP, geranylgeranyl diphosphate; GGOH, geranylgeraniol; FPP, farnesyl diphosphate; GPP, geranyl diphosphate; IPP, isopentenyl diphosphate.

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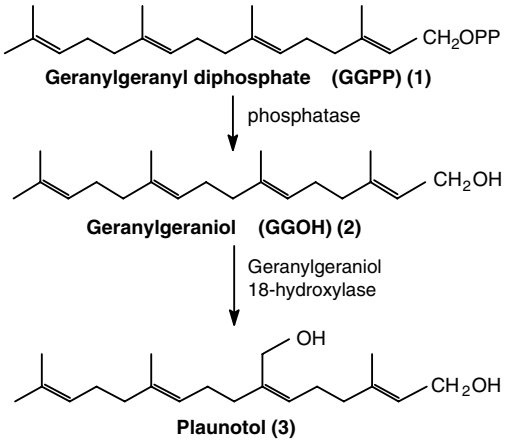

Fig. 1. Proposed biosynthetic pathway of plaunotol (**3**).

this anti-peptic drug (Kelnac®) (Ogiso et al., 1985). Plaunotol (**3**) has also been shown to accumulate mainly in chloroplasts of *C. stellatopilosus* (Sitthithaworn et al., 2001, in press). Feedings of [U-^{13}C] glucose and of [1-^{13}C] glucose into cut shoots of the plant have revealed that all four isoprene units of plaunotol (**3**) have the same labeling pattern, which is also consistent with its exclusive acquisition via the deoxyxylulose phosphate pathway (Wungsintaweekul and De-Eknamkul, 2005).

Although the acyclic diterpenoids have been well recognized to be present in algae (Daoudi et al., 2001; Amico et al., 1980) and plants (Ogiso et al., 1978; Pattenden and Jondiko, 1989), very little is known about the enzymes and genes involved in the key step of the conversion of geranylgeranyl diphosphate (**1**) (GGPP), into this particular group of linear terpenoid compounds. This is in contrast with the group of cyclic diterpenoids in which a number of diterpenoid synthases (cyclases) have been purified and extensively studied (for review, see Dewick, 2002).

In our previous work on the biosynthetic study of plaunotol in *C. stellatopilosus*, a sequential conversion of GGPP (**1**) → GGOH (**2**) → plaunotol (**3**) as well as enzyme activities of GGPP phosphatase and GGOH-18-hydroxylase were detected in a reaction mixture containing the 20,000*g* pellet fraction obtained from cell-free extracts of the leaves (Tansakul and De-Eknamkul, 1998). This has suggested that plaunotol (**3**) might be biosynthesized from GGPP (**1**) via two steps of reactions: dephosphorylation of GGPP (**1**) to form GGOH (**2**) and 18-hydroxylation of GGOH (**2**) to form plaunotol (**3**) (Fig. 1). In order to confirm that this specific pathway is present in *C. stellatopilosus*, we aimed to identify and characterize the two membrane-bound enzymes detected. Characterization of the GGOH-18-hydroxylase enzyme has been reported previously (Tansakul and De-Eknamkul, 1998). In this paper, we report the results of purification and characterization of GGPP phosphatase from *C. stellatopilosus* leaves.

2. Results

2.1. TLC-densitometric assay and solubilization of membrane-bound GGPP phosphatase

In this study, the technique of non-radioactive TLC-densitometry was developed for determining the catalytic activity of GGPP phosphatase in young leaves of *C. stellatopilosus*. The green pellet fraction obtained from 20,000*g* centrifugation of a crude enzyme extract was used as an enzyme source, and non-radioactively labeled GGPP was used as substrate. The reaction was terminated by adding ethyl acetate which could extract the reaction product, GGOH (**2**), from the reaction mixture. Separation of the organic phase by TLC followed by densitometric scanning of the plate under 210 nm gave a result of a well-separated TLC-chromatogram for quantitating the formation of GGOH (**2**). As shown in Fig. 2A, GGOH (**2**) was clearly detected as a sharp peak by the densitometer; absolutely no GGOH (**2**) peak was detected in either the boiled control or the reaction mixture without the substrate. This TLC-densitometric technique was, therefore, used as standard assay for determining the enzyme activity of GGPP phosphatase.

When the same 20,000*g* pellet was solubilized with 0.1% Triton X-100 and followed by 100,000*g* centrifugation, it was found that the GGPP phosphatase activity originally present in the 20,000*g* pellet was mostly recovered in the solubilized fraction of the 100,000*g* supernatant (Fig. 2B).

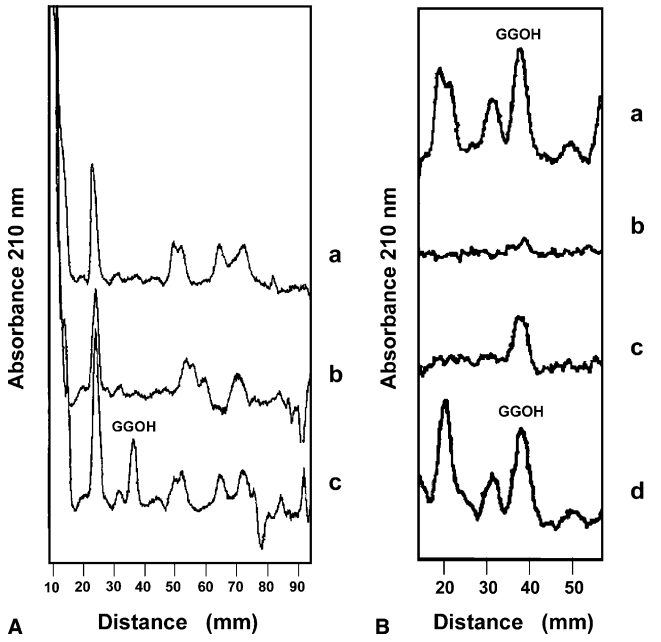

Fig. 2. (A) Typical TLC-chromatograms of GGPP phosphatase assay of (a) boiled control, (b) absence of substrate and (c) complete reaction. (B) TLC-densitometric chromatograms of GGPP phosphatase activity assayed before (a, pellet; b, supernatant) and after (c, pellet; d, supernatant) membrane solubilization by 0.1% Triton X-100 followed by 100,000*g* centrifugation.

Only less than 20% of the total phosphatase activity was left in the pellet fraction after the detergent treatment. This clearly showed that GGPP phosphatase could be removed from the membrane part of the 20,000*g* green pellet without significant loss of the enzyme activity. Hence, the GGPP phosphatase activity solubilized from the 100,000*g* pellet was subjected to further enzyme purification.

Preliminary studies on the enzyme stability, optimum pH and optimum temperature, were first carried out with the crude enzyme extracts for proper handling of enzyme preparations during purification. It was found that the enzyme solution stored at 4 °C lost its activity completely in 10 days, whereas, at −20 °C storage, the enzyme still remained fully active for at least 45 days of the monitoring (Fig. 3A). The catalytic activity of the crude enzyme extract appeared to be optimal at pH 7.0. By using 150 mM Tris–HCl, pH 7.0 in the reaction mixture, the formation of GGOH (**2**) from GGPP (**1**) showed linearity with time for at least 60 min. The phosphatase showed a wide range of optimum temperature for catalytic activity, from 40 to 70 °C (Fig. 3B).

2.2. Enzyme purification

The solubilized GGPP phosphatase extract prepared from 2 kg fresh leaves was first applied onto a BioGel A gel filtration column. This step of size exclusion chromatography allowed the solubilized phosphatase activity in the crude extract be separated into two peaks, designated PI and PII (Fig. 4A). PI was eluted as green fractions, whereas PII was eluted as clear solution with higher GGPP phosphatase activity. The pooled active fractions of both PI and PII were purified separately using another gel filtration column of Superose 6. Under the conditions of this column, PI activity appeared to be eluted as a single peak of protein with the eluting volume of 57 ml (Fig. 4B), and PII activity was eluted as a mixture of protein peaks with the volume of 73 ml (Fig. 4C). The resulting active PI fractions were pooled and kept at −20 °C for enzyme characterization, whereas the active PII fractions were pooled and subjected to the last purification step of UNO Q anion-exchange chromatography (Fig. 4D) before performing characterization of the enzyme.

As summarized in Table 1, the purification procedure gave PII about 270-fold purification with about 4% recovery of the enzyme activity and gave PI about 3-fold purification with 21% recovery. The specific activity of the purified PI preparations varied from 0.08 to 0.10 nkat mg^{-1}, whereas that of PII preparations varied from 6.0 to 8.0 nkat mg^{-1}. The purity of the final PI and PII preparations was checked by SDS–PAGE as compared with the protein patterns of other preparations obtained from the sequential steps of the chromatography. As shown in Fig. 5, the purified PI preparation migrated as a major single band at M_r 58,000 whereas PII preparation showed a clear protein band at M_r 30,000.

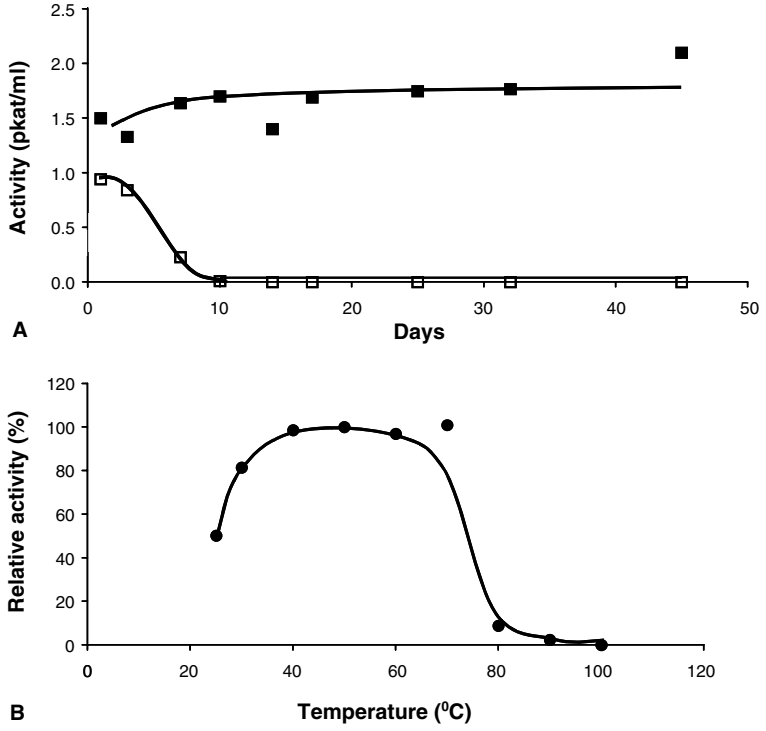

Fig. 3. (A) Stability of *C. stellatopilosus* GGPP phosphatase activity during the storage at −20 (■) and 4 °C (□). (B) Effect of temperature on the catalytic activity of GGPP phosphatase activity.

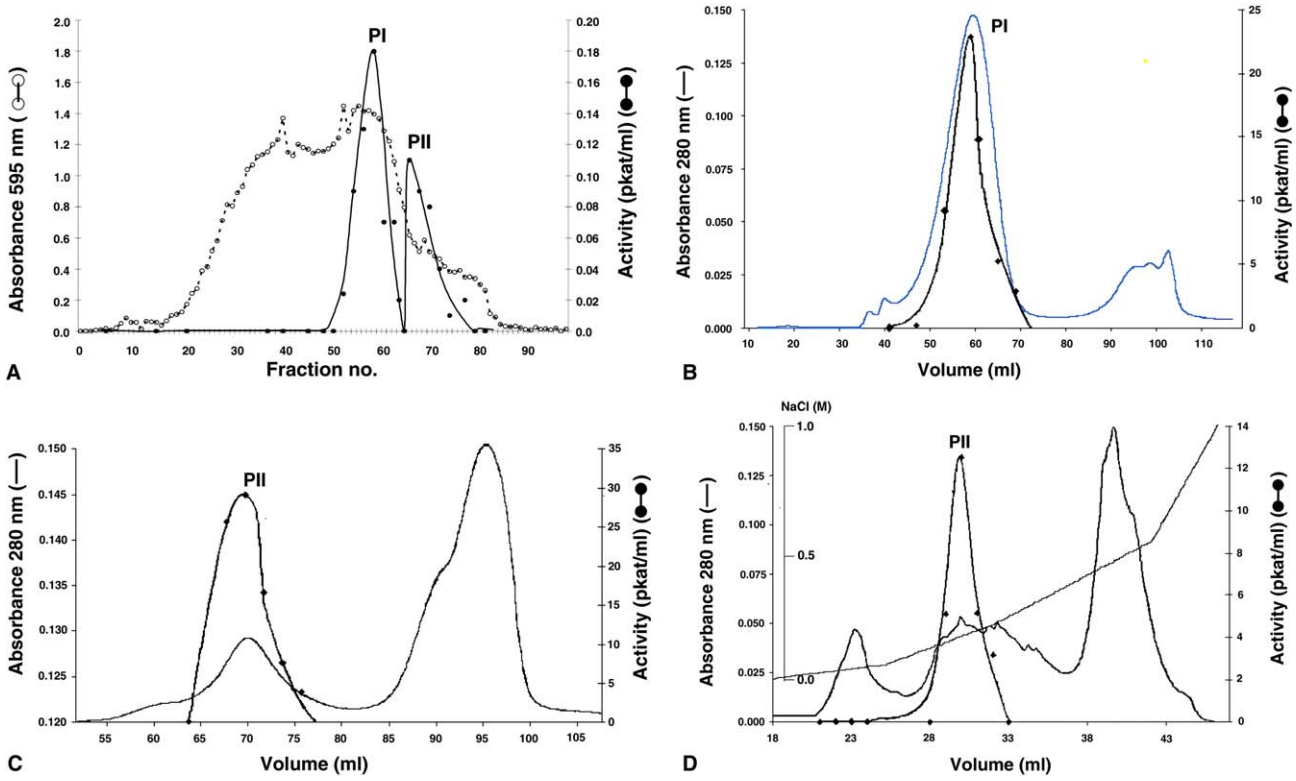

Fig. 4. (A) Separation of GGPP phosphatase activity in the crude extract into two peaks of PI and PII by BioGel A gel filtration chromatography, followed by Superose 6 gel filtration of PI (B) and PII (C). PII was finally purified by UNO Q anion-exchange chromatography (D).

Table 1
Summary of GGPP phosphatase purification from *C. stellatopilosus* leaves

Purification step	Volume (ml)	Total protein (mg)	Total activity (nkat)	Specific activity (nkat mg^{-1})	Yield (%)	Purification factor (-fold)
Crude extract	228	119.8	2.52	0.02	100	1.0
BioGel A						
PI	50	27.0	0.80	0.03	32	1.4
PII	40	2.32	1.12	0.49	45	23
Superose 6						
PI	100	7.8	0.52	0.07	21	3
PII	24	0.17	0.56	3.32	23	157
UNO Q						
PII	12	0.016	0.092	5.79	4	274

2.3. Enzyme characterization

2.3.1. Molecular properties of PI and PII

The native molecular masses of PI and PII were determined by employing Superose 6 pre-equilibrated gel filtration column with thymoglobulin (670 kDa), gamma-globulin (158 kDa), ovalbumin (44 kDa) and myoglobulin (17 kDa) as standard proteins. It was found that PI was eluted at a volume corresponding to a protein size of 232 kDa whereas PII appeared to have the size of 34 kDa. Under denaturing conditions of SDS–PAGE, the estimated molecular weight of PI and PII appeared to be 58.7 kDa and 30.6 kDa, respectively (Fig. 5). These results suggested that PI is a tetrameric protein of 58 kDa subunits, whereas PII is a monomeric protein of 30–34 kDa.

2.3.2. Catalytic properties

The pH optimum for catalytic activity of the two phosphatases was found to be 6.0–6.5 for PI and 6.5–7.0 for PII (Fig. 6). For substrate specificity, both PI and PII appeared to be able to utilize GGPP, FPP and GPP as their substrates although GGPP showed considerable preference (Table 2). No activity was detected when IPP was used as substrate. Kinetic studies under the standard assay conditions (Fig. 7) showed that the apparent K_m value of PI for GGPP was 0.2 mM, and V_{max} was 0.28 nkat mg^{-1}.

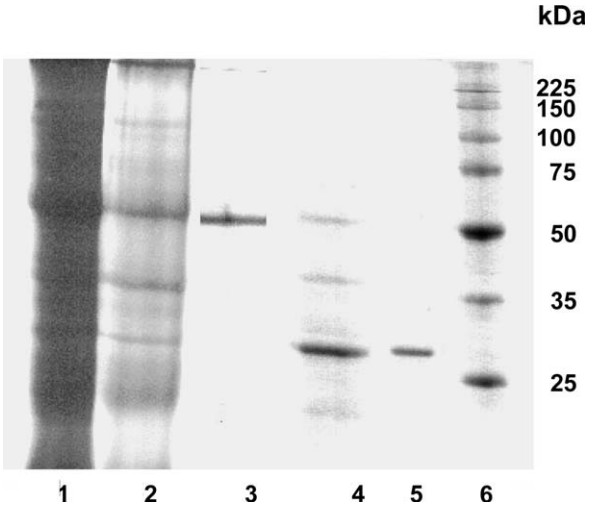

Fig. 5. SDS–PAGE of various enzyme fractions obtained during enzyme purification of GGPP phosphatases from *C. stellatopilosus*. Lane 1, membrane-solubilized extract; lane 2, BioGel A of PI; lane 3, Superose 6 of PI; lane 4, BioGel A of PII; lane 5, Superose 6 of PII and lane 6, marker proteins and molecular mass ($\times 10^{-3}$).

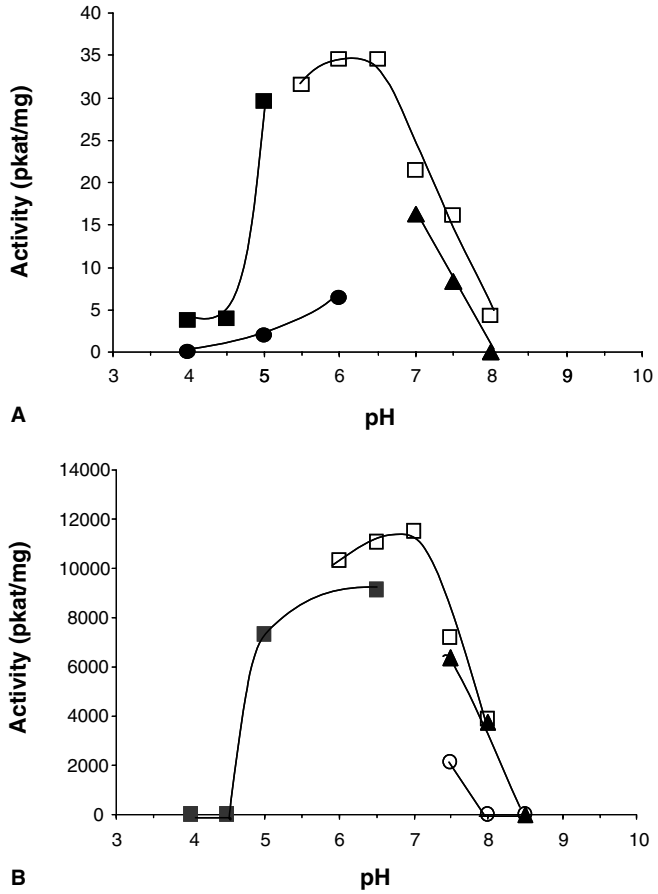

Fig. 6. Effect of pH on GGPP phosphatase activity of PI (A) and PII (B). (●) Na_2HPO_4/citric acid, (□) MOPS buffer, (▲) Tris–HCl buffer, (○) glycine buffer, (■) citric acid/sodium citrate.

No substrate inhibition of PI was observed with the concentration up to 0.4 mM. (Fig. 7A). For PII, its apparent K_m for GGPP was 0.1 mM, and V_{max} was 7.5 nkat mg^{-1}.

Table 2
Substrate specificity of PI and PII

Substrate (0.2 mM)	% Relative activity	
	PI	PII
GGPP	100[a]	100[b]
FPP	11	23
GPP	7	10
IPP	0	0

[a] Specific activity of PI was 0.03 nkat mg^{-1} (100%).
[b] Specific activity of PII was 1.4 nkat mg^{-1} (100%).

Table 3
The influence of metal ions on *C. stellatopilosus* GGPP phosphatase activities of PI and PII

Substance (1.0 mM)	% Relative activity	
	PI	PII
Boiled control	0	0
No substance	100[a]	100[b]
$MgCl_2$	117	105
$ZnSO_4$	103	0
$MnSO_4$	96	0
$CoCl_2$	79	0
KCl	82	111
NaCl	77	95
Na_2MoO_4	0	0

[a] Specific activity of PI was 0.02 nkat mg^{-1} (100%).
[b] Specific activity of PII was 0.2 nkat mg^{-1} (100%).

Substrate inhibition was observed with PII when the concentration of GGPP was higher than 0.2 mM (Fig. 7B).

2.4. Effects of metal ions on GGPP phosphatase activities

The influence of metal ions on the enzyme activities of PI and PII was also determined. Using 1.0 mM concentration of various salts under the standard assay, it was found that the two forms of GGPP phosphatases were affected differently by various metal ions. As shown in Table 3, the divalent ions of Zn^{2+}, Mn^{2+} and Co^{2+} had no significant effect on PI activity but completely inhibited PII activity. Na^+ and K^+ showed slightly decreased the PI activity but had no effect on that of PII. Only MoO_4^{2-} showed similar inhibitory effect for both PI and PII.

3. Discussion

The results achieved in this work indicate the presence of two forms of membrane-bound GGPP phosphatase enzyme in leaves of *C. stellatopilosus*. The membrane fraction is part of the green pellets obtained from 20,000*g* centrifugation of leaf cell-free extracts. Chloroplast membrane is obviously present in this starting preparation. As a matter of fact, plaunotol (**3**) has been reported to accumulate in chloroplasts of *C. stellatopilosus* leaves (Sitthithaworn et al., 2001), and to be composed of the isoprene units that are formed exclusively from the chloroplast's deoxyxylulose

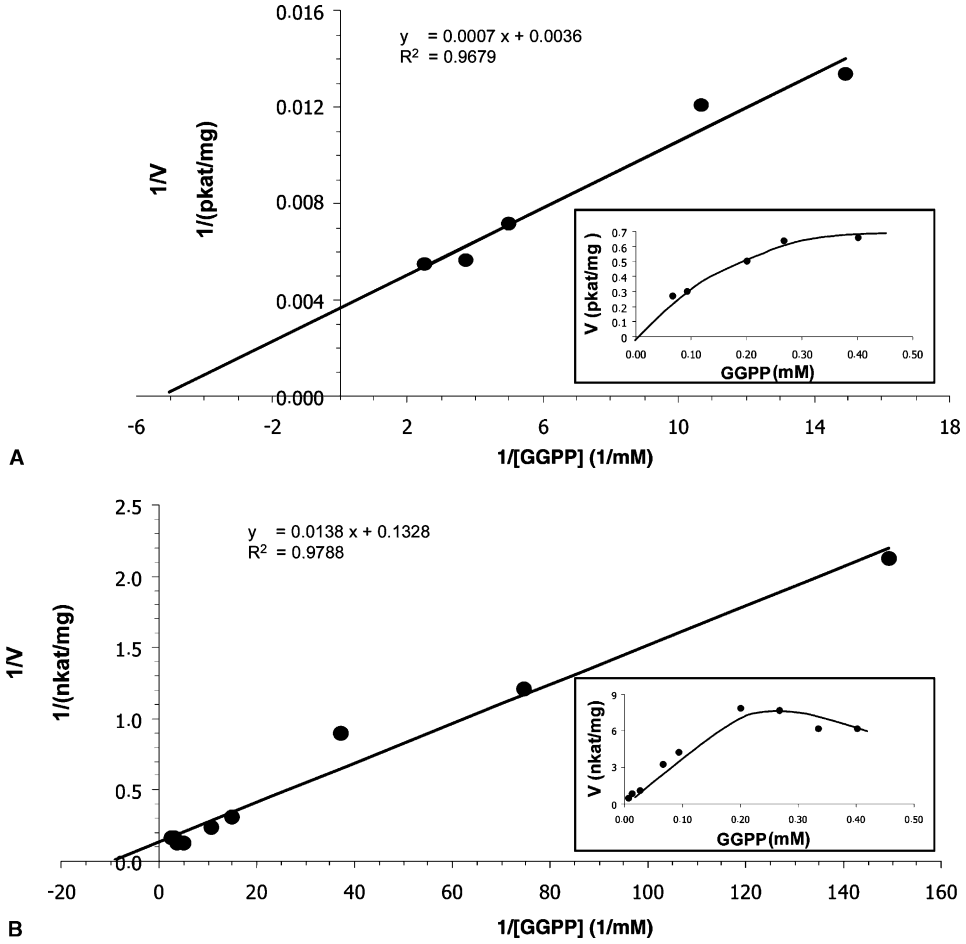

Fig. 7. Lineweaver–Burk plots of GGPP phosphatase activity of PI (A) and PII (B) against GGPP concentration. (Inset) Michaelis–Menten plot.

phosphate pathway (Wungsintaweekul and De-Eknamkul, 2005). Therefore, it is likely that the whole biosynthetic machinery of plaunotol (**3**), including GGPP phosphatase enzyme, is located in the chloroplasts of this plant.

As membrane-bound enzymes, it appears that the two phosphatase forms, PI and PII, can be removed from their membrane system using 0.1% Triton X-100 without significant loss of enzyme activities. The resulting greenish enzyme preparations still contain a number of protein bands as shown by SDS–PAGE. However, because of their considerable difference in size, PI and PII can be readily separated from each other by the first step of gel filtration using Bio-Gel A column. This type of gel has a wide protein separation range (10–15,000 kDa) and allows Triton X-100 remaining in the crude extract be removed from the enzyme fractions. This initial separation of the two phosphatase activities allows PI and PII be purified separately in subsequent steps. The enzyme activities of both PI and PII are very stable upon storage at −20 °C. No significant loss of the activities was observed during 45 days of the monitoring. This allows freezing and thawing of active enzyme fractions be performed during enzyme purification.

By using another gel filtration column of Superose 6 (5–5000 kDa separation range), PI was eluted nicely as a single peak corresponding to the molecular mass of 232 kDa whereas PII is eluted as a minor protein with 34 kDa. Under SDS–PAGE, PI preparation appears to be clean enough for enzyme characterization whereas PII needs another step of anion-exchange chromatography before its characterization. The purification procedure gives PI 3-fold purification with 21% recovery and PII 270-fold with 4% recovery.

Enzyme characterization using the purified PI and PII preparations is summarized in Table 4. It is clear that the

Table 4
Some properties of PI and PII with GGPP phosphatase activity

Enzyme property	PI	PII
Molecular mass		
Native (kDa)	232	34
Subunit (kDa)	58	30
Optimum pH	6.0–6.5	6.5–7.0
K_m for GGPP (mM)	0.2	0.1
GOH, FOH, GGOH effect	No	No
Substrate specificity	GGPP:FPP:GPP (100:11:7)	GGPP:FPP:GPP (100:23:10)
Substrate (GGPP) inhibition	No	Yes
Ion inhibition	MoO_4^{2-}	MoO_4^{2-}, Zn^{2+}, Mn^{2+}, Co^{2+}

two phosphatases have different size and subunit structure. PI (232 kDa, 4 subunits) is much bigger than PII (30–34 kDa, monomeric). Both catalyze the same dephosphorylation reaction with similar properties of preference of substrates (GGPP > FPP > GPP), activity-independence of Mg^{2+} and complete inhibition by MoO_4^{2-}. On the other hand, PI and PII appear to be different in their kinetics, pH optimum, substrate inhibition and, in particular, the effect of divalent metal ions. PII is strongly inhibited by Mn^{2+}, Zn^{2+} and Co^{2+} whereas PI is not affected. In the literature, there have been a few reports on the allyl phosphatases/pyrophosphatases, including rice GGPPase and FPPase (Nah et al., 2001), orange prenylphosphatases (Perez et al., 1980), rat GGPPase and FPPase (Bansal and Vaidya, 1994), calf dolichyl-PPase (Scher and Waechter, 1984) and pig dolichyl phosphatase (Frank and Waechter, 1998). Almost all of these phosphatases are membrane-bound enzymes and have no information on their molecular masses. However, by comparing the properties of PI and PII with these reported phosphatases, it appears that both have no particular similarity to either one of the enzymes.

Based on the catalytic reaction, *C. stellatopilosus* phosphatases PI and PII seem to be closely related to the enzyme group of prenyldiphosphatases (EC 3.1.7.1: prenyl diphosphate → prenol + diphosphate). For this group, the enzymes geranylgeranyl and farnesyl diphosphatases from rice seedings have been reported (Nah et al., 2001). These diphosphatases appear to be induced by UV-C irradiation and located in the microsomal fraction. Its optimal pH is 7.9. On the other hand, another group of acid phosphatases (EC 3.1.3.2: a phosphate monoester + H_2O → an alcohol + phosphate) has been reported to be present in the flavedo of *Citrus sinensis* (Perez et al., 1980). These enzymes appear to have prenylphosphatase activities to hydrolyze a wide range of C10–C20 allylic diphosphates (GPP, FPP, GGPP) in a sequential manner to their corresponding monophosphates and prenyl alcohols. Obviously, the main difference between these two phosphatase groups is the catalytic process of the diphosphate dephosphorylation: a one-step pyrodephosphorylation for the prenyl diphosphatases and a successive monodephosphorylation catalyzed by the prenyl diphosphate phosphatases. Our preliminary studies based on in vivo chloroplast feedings with [1-^{3}H]GGPP and [1-^{3}H]GGMP and in vitro enzyme catalyzed reactions have suggested that the formation of GGOH from GGPP proceeds via two successive monodephosphorylation reactions (Nualkaew et al., 2005). The findings of both GGPP phosphatases in this study and GGOH-18-hydroxylase reported previously (Tansakul and De-Eknamkul, 1998) have suggested their involvement in the biosynthetic pathway of plaunotol in *C. stellatopilosus*, although it might be also possible that the phosphatase is simply involved in the putative recycling cycle for the prenyl phosphates as described previously by Waechter group (Thai et al., 1999).

4. Experimental

4.1. Plant material

Fresh leaves of *C. stellatopilosus* were collected from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok, Thailand. A voucher specimen is deposited in the Herbarium Royal Forest Department in Bangkok, Thailand under No. 21867.

4.2. Enzyme extraction

Fresh leaves (2 kg) were washed and ground using pestle and mortar in the presence of liquid nitrogen. After this, all steps were performed at 4 °C. The fine powder was homogenized for 20 min by stirring in 4 l Buffer A (83 mM tricine/NaOH pH 7.8 containing 5 mM β-mercaptoethanol, 10 mM EDTA, and 10 mM $MgCl_2$). After filtering through four-layer cheesecloth, the crude homogenate was centrifuged at 3000*g* for 10 min to discard cell debris. The crude supernatant was then centrifuged at 20,000*g* for 20 min and the pellet part was collected. The pellet was washed with Buffer A for two times before being dissolved with 100 ml Buffer A and solubilized with 0.1% Triton X-100, mixed thoroughly, and centrifuged at 100,000*g* for 1 h to obtain a green supernatant fraction which was used as crude GGPP phosphatase extract.

4.3. Enzyme purification

The crude enzyme extract was concentrated to 10 ml by Centriprep-10 before being applied onto BioGel A column. The column was pre-equilibrated with Buffer B (100 mM tricine/NaOH pH 7.8 containing 5 mM β-mercaptoethanol, and 1 mM EDTA). It was then eluted with Buffer B at a flow rate 0.5 ml/min and fractions of 5 ml each were collected until no protein was detected. The fractions showing two activities of GGPP phosphatase, PI and PII, were pooled separately and concentrated by Centriprep-10. The concentrated fractions of PI and PII were then applied separately onto another gel filtration column of Superose 6 which had been equilibrated with Buffer C (20 mM Tris–HCl pH 7.8 containing 5 mM β-mercaptoethanol, and 1 mM EDTA). The active fractions of PI was pooled and kept at −20 °C for enzyme characterization whereas the active fractions of PII from Superose 6 column were pooled and applied to UNO Q column pre-equilibrated with Buffer C. The column was washed with Buffer C and eluted with Buffer C containing NaCl gradient from 0 to 1 M to obtain the final enzyme preparation of PII.

4.4. GGPP phosphatase assay

The enzyme activity of GGPP phosphatase was determined by means of TLC-densitometry. Each enzyme solution was incubated in the standard reaction mixture

containing 150 mM Tris–HCl pH 7.0, 67 μM GGPP, and 100 μl enzyme solution in a total volume of 150 μl. After incubation at 30 °C for 60 min, the reaction mixture was extracted twice with 300 μl EtOAc. The pooled EtOAc was dried, redissolved and subjected to TLC (silica gel GF254) in Benzene–EtOAc (9:1). The TLC plate was then scanned by a TLC densitometer (wavelength 210 nm). The amount of enzymatic product formed was estimated from the area under the peak of GGOH (R_f 0.27) and the calibration curve of authentic GGOH which showed linearity between 2.0 and 20 nmol of GGOH.

4.5. Analytical procedure

SDS–polyacrylamide gel electrophoresis with 12% gel was performed according to the method of Laemmli (1970). Protein concentrations were measured by protein assay reagent (Bio-Rad) in microplate at 595 nm, and using BSA as standard.

4.6. Enzyme characterization

Enzyme characterization was performed under the conditions of the standard assay. The optimum pH for GGPP phosphatase activity was determined by using various buffers (150 mM) with different pH range: citric acid–sodium citrate, NaH_2PO_4–citric acid, MOPS–NaOH, tricine–NaOH, Tris–HCl, and glycine–NaOH. The effect of metal ions was tested with 1.0 mM of various metal ion solutions ($MnSO_4$, $CoCl_2$, Na_2MoO_4, $ZnSO_4$, $MgCl_2$). Substrate specificity of PI and PII was determined by using 0.2 mM of IPP, GPP, or FPP and GGPP (all from Sigma). Boiled control of each substrate was used as the blank of reaction. The values of K_m and V_{max} for PI and PII were determined under the standard assay conditions with different concentrations of GGPP and determined by using Lineweaver–Burk plot (see Fig. 7).

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