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Geranylgeraniol formation in *Croton stellatopilosus* proceeds via successive monodephosphorylations of geranylgeranyl diphosphate

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Abstract—The process of catalytic dephosphorylation of geranylgeranyl diphosphate (GGPP) to give geranylgeraniol (GGOH) in *Croton stellatopilosus* leaves was examined by in vivo chloroplast feedings with [1-^{3}H]GGPP and [1-^{3}H]GGMP and in vitro enzyme-catalyzed reactions. The results strongly suggest that the formation of GGOH from GGPP proceeds in the chloroplasts via two successive monodephosphorylation reactions. Hence, we name the enzyme geranylgeranyl diphosphate phosphatase rather than geranylgeranyl diphosphatase based on its catalytic mechanism.
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Diterpenes are a group of structurally diverse C_{20} terpenoid compounds which are all derived from geranylgeranyl diphosphate (GGPP). There are two basic groups, acyclic and cyclic diterpenoids, which are highly variable in structure and are classified in accordance with their biogenetic origin.[1] Between the two groups, the cyclic diterpenoids have received much more attention with respect to the studies of enzymes and genes that are involved in the key step of the conversion of GGPP into various cyclic diterpenoid compounds. To date, a number of diterpenoid cyclases/synthases have been purified and extensively studied.[2] On the other hand, for the acyclic diterpenoids, very little is known about the enzymes and genes that convert GGPP into this group of linear C_{20} compounds.

Among the acyclic diterpenoids, plaunotol which is one of the simplest derivatives of geranylgeraniol (GGOH) is the subject of our interest. This 18-hydroxy GGOH derivative has been found to be present in a Thai medicinal plant of *Croton stellatopilosus* Ohba[3] (known previously as *C. sublyratus* Kurz[4]) and has been used as an anti-peptic ulcer drug under the commercial name of Kelnac®.[5] Plaunotol has been found to accumulate mainly in chloroplasts of *C. stellatopilosus*.[6] It has been shown recently to be biosynthesized via the deoxyxylulose phosphate pathway.[7] Our previous work on the biosynthesis of plaunotol has shown that there are two enzyme activities, GGPP phosphatase and GGOH-18-hydroxylase, present in the cell-free extracts prepared from the leaves of *C. stellatopilosus*.[8] This suggested that GGPP is first dephosphorylated to form GGOH which is then hydroxylated at C-18 to form plaunotol (Fig. 1).

In this study, the catalytic dephosphorylation of GGPP was examined to determine whether it occurs via a one-step pyrodephosphorylation or by two successive monodephosphorylation reactions. In doing this, we first prepared radioactively labelled [1-^{3}H]GGMP and [1-^{3}H]GGPP from [1-^{3}H]GGOH. This was carried out by enzymatic reactions using microsomal preparations obtained from *Nicotiana tabacum* cell cultures. The microsomal fraction has been shown to be capable of catalyzing two successive monophosphorylations of [1-^{3}H]GGOH to form [1-^{3}H]GGMP and [1-^{3}H]GGPP.[9] A reaction mixture containing 50 μg of microsomal

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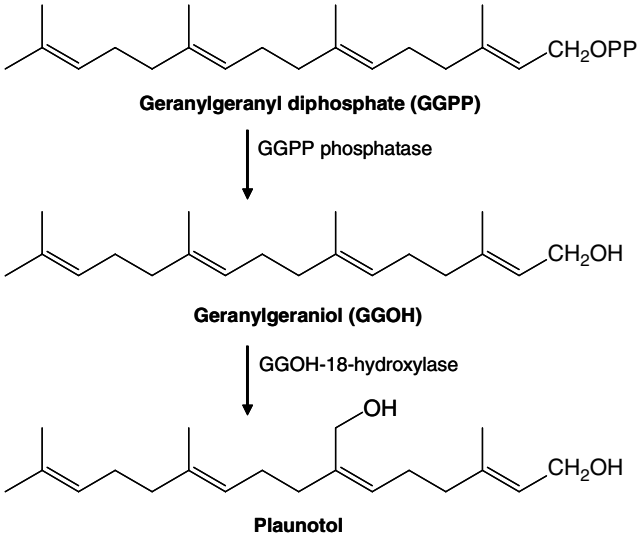

Figure 1. Proposed biosynthetic pathway of plaunotol.

protein of *N. tabacum*, 0.2 μCi [1-^{3}H]GGOH, 62.5 mM MOPS buffer pH 6.5, 6.25 mM cytidine triphosphate, 10 mM sodium orthovanadate and 5 mM $MgCl_2$ in a total volume of 80 μL (130 tubes) was incubated for 60 min at 37 °C. Under these conditions, both [1-^{3}H]GGMP and [1-^{3}H]GGPP appeared to be formed in the reaction mixture with 53–60% conversion of [1-^{3}H]GGOH (Fig. 2A). In addition to *N. tabacum*, we found that *C. stellatopilosus* microsomal fractions prepared from the leaves could also catalyze the same successive monophosphorylation reactions to generate [1-^{3}H]GGMP and [1-^{3}H]GGPP (Fig. 2B). The labelled products were then separated from each other by using a reversed phase column of MCI gel CHP-20P. The eluted [1-^{3}H]GGPP and [1-^{3}H]GGMP (specific activity 28 mCi/mmol each) were characterized using alkaline phosphatase enzyme (Sigma) which converted both labelled products back to [1-^{3}H]GGOH with identical TLC characteristics to the authentic compound.

In performing feeding experiments using intact chloroplasts, fresh leaves (10 g) of *C. stellatopilosus* were cut into small pieces and suspended in a mixture of 50 mL chloroplast-isolation buffer (50 mM HEPES–KOH, pH 8.0, 1.0 mM EDTA, 1.0 mM 1,4-dithioerythritol and 0.4 M sucrose) and macerated in a blender for

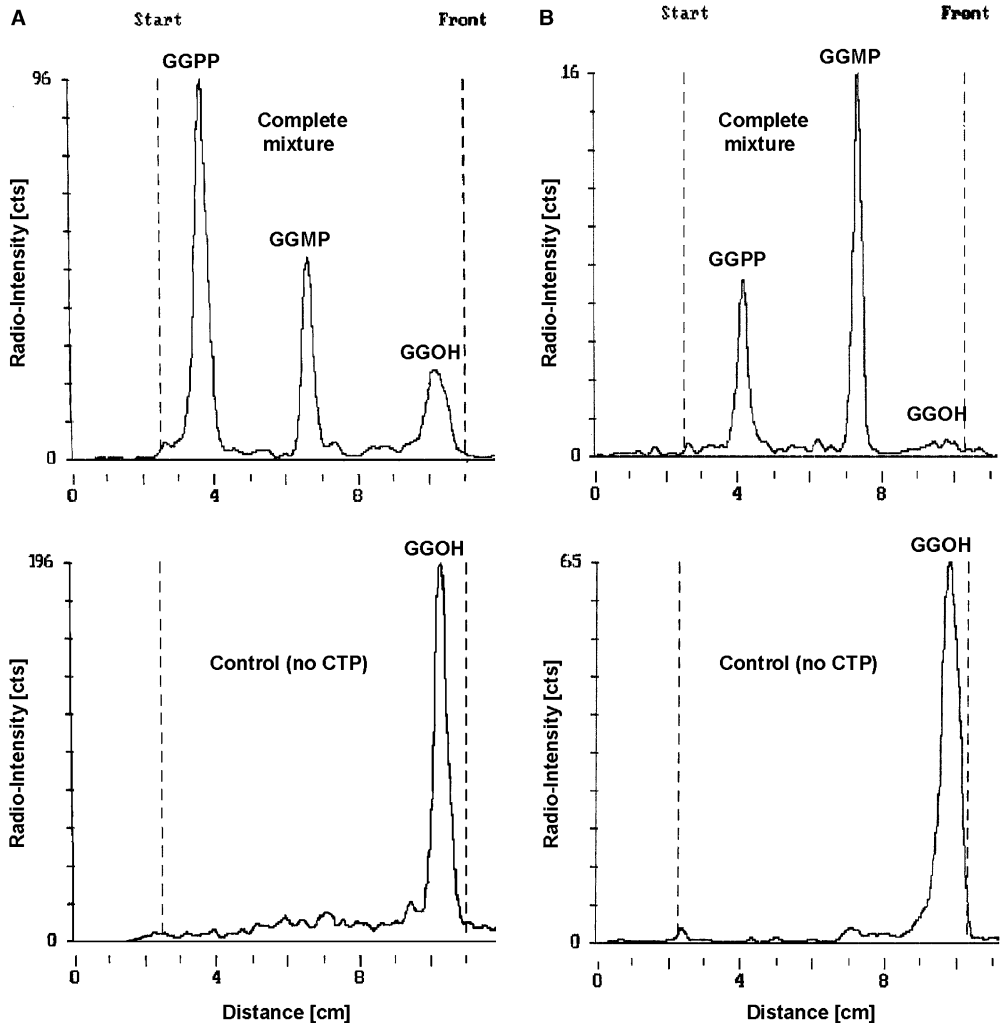

Figure 2. TLC-radiochromatograms of the enzymatic products obtained from the conversion of [1-^{3}H]GGOH in the presence and absence of cytidine triphosphate (CTP) by microsomal preparations of (A) *Nicotiana tabacum* cell cultures and (B) *Croton stellatopilosus* leaves.

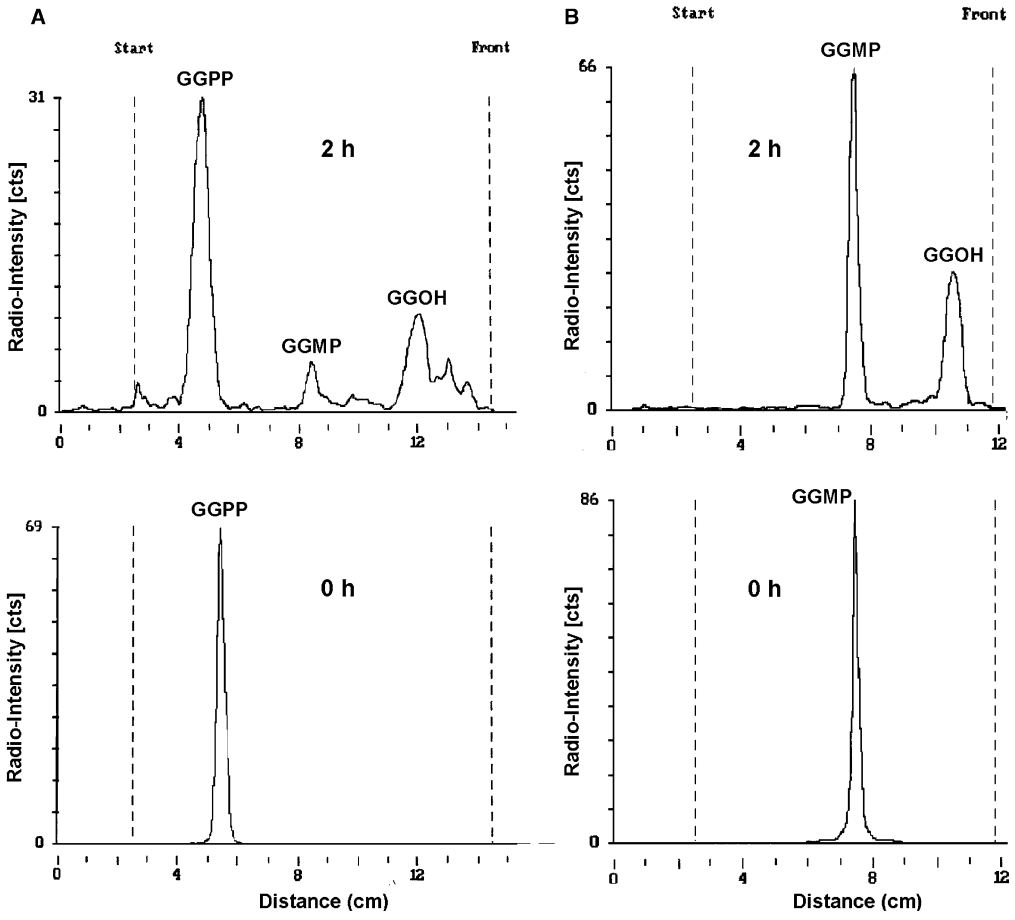

Figure 3. TLC-radiochromatograms of chloroplast extracts obtained after feeding *C. stellatopilosus* chloroplasts with (A) [1-^{3}H]GGPP and (B) [1-^{3}H]GGMP for 2 h.

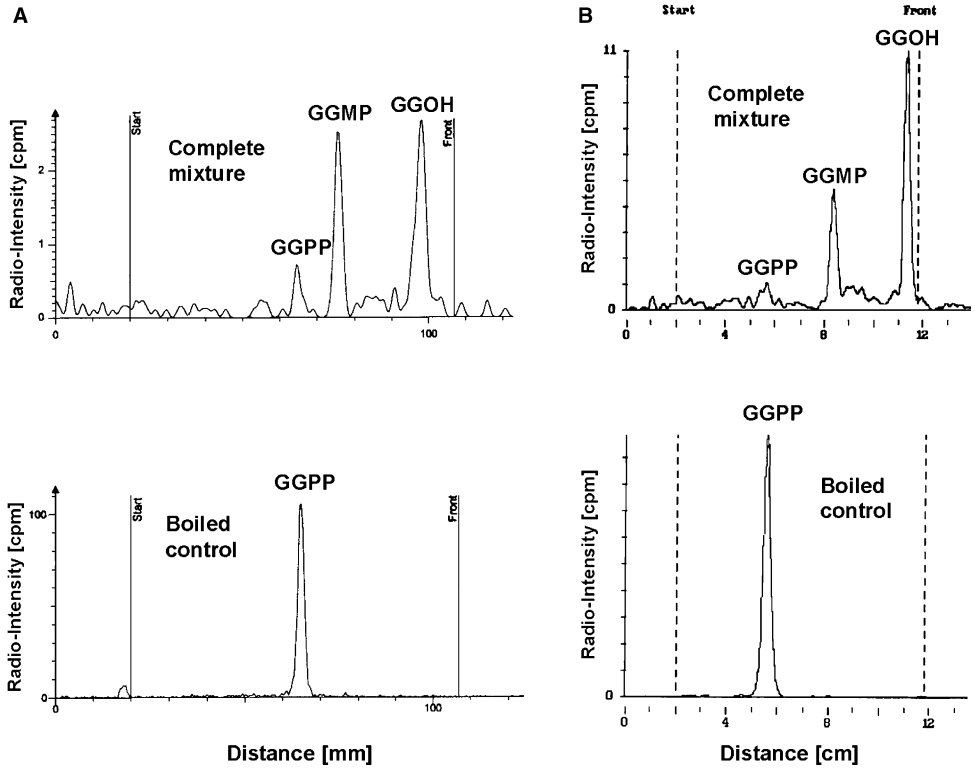

Figure 4. TLC-radiochromatograms of the enzymatic reactions of [1-^{3}H]GGPP conversion catalyzed by *C. stellatopilosus* GGPP phosphatases of (A) PI preparation and (B) PII preparation.

30 s. The suspension was filtered through four layers of nylon gauze and centrifuged at 1500*g* for 10 min. The resulting pellet was resuspended with a small volume of 50 mM HEPES–KOH, pH 7.6 and 0.1 mM 1,4-dithioerythritol and was layered over a sucrose 15–70% step gradient in the same buffer solution. Chloroplasts were collected at the interface between 30% and 40% after centrifugation for 60 min at 50,000*g*. The chloroplast fraction was diluted with the same buffer to obtain a final sucrose concentration of 15% and was used as an intact chloroplast preparation. ^{3}H-labelled GGPP samples were then fed to the prepared intact chloroplasts at a concentration of 70 μM (0.1 μCi) for 2 h at 30 °C. The suspension was then dissolved with ethyl acetate and methanol (1:1), concentrated and was subjected to TLC-radioscan analysis. The label from [1-^{3}H]GGPP was detected in the molecules of GGMP and GGOH during the feeding (Fig. 3A). Similarly, feeding of the intermediate [1-^{3}H]GGMP to the intact chloroplasts also showed conversion of the monophosphate to [1-^{3}H]-GGOH (Fig. 3B). These results clearly suggested that GGPP was utilized in this organelle by two successive monodephosphorylation reactions in forming GGOH.

In order to confirm this observation, enzymatic studies on *C. stellatopilosus* phosphatase utilizing GGPP were performed. Green pellets obtained from 20,000*g* centrifugation of leaf cell-free extracts were previously shown to possess GGPP phosphatase activity.[8] In this study, the activity detected was found to be closely associated with the membrane fraction and could be solubilized by 0.1% Triton X-100 without significant loss of the enzyme activity. Purification of the solubilized enzyme preparation revealed that the GGPP phosphatase possessed two activity peaks which could be separated by an initial step of gel filtration chromatography using BioGel A. The two activities, designated PI and PII, were purified further using a combination of gel filtration and anion-exchange chromatography to afford partially purified enzyme preparations with 3-fold and 270-fold purification, respectively.[10]

With the purified preparations of PI and PII in hand, the catalytic mechanism of GGPP phosphatase was studied in a reaction mixture containing 0.1 μCi [1-^{3}H]GGPP, 150 mM Tris/HCl, pH 7.0 and the purified enzyme fraction in a total volume of 50 μL. The reaction was incubated for 8 h at 30 °C and subjected to detection for product formation. It was found that under these conditions, both PI and PII preparations showed similar TLC-radioscan patterns with the presence of [1-^{3}H]GGMP in the reaction mixture (Fig. 4A and B). Again, when [1-^{3}H]GGMP was used as the substrate instead of [1-^{3}H]GGPP, it appeared that the monophosphate substrate could also be converted to GGOH by both phosphatase enzymes (Fig. 5). This confirmed that GGPP was dephosphorylated at the enzyme level by two successive monodephosphorylations in forming GGOH.

The consistent results of both the in vivo chloroplast feeding with [1-^{3}H]GGPP and [1-^{3}H]GGMP and the in vitro enzyme-catalyzed reactions using the two labelled substrates strongly indicate the involvement of

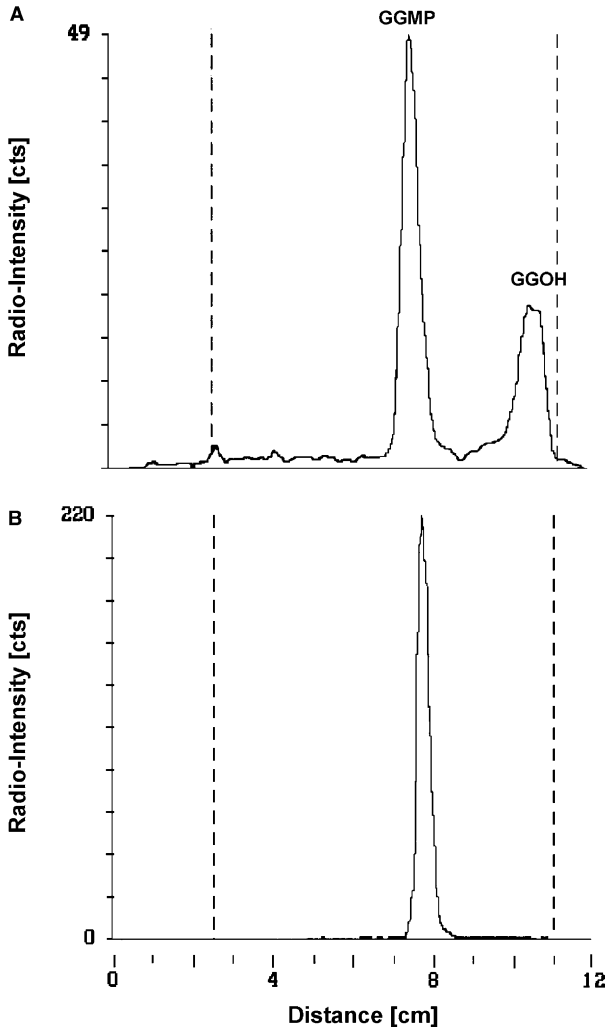

Figure 5. TLC-radiochromatograms of the enzymatic reaction catalyzed by the PII preparation with [1-^{3}H]GGMP as substrate. Similar chromatograms were obtained with the PI preparation.

two successive monodephosphorylation reactions in the conversion of GGPP to GGOH in *C. stellatopilosus*. Thus, the enzyme catalyzing this reaction was named geranylgeranyl diphosphate phosphatase rather than geranylgeranyl diphosphatase.

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