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POLYPRENYL DIPHOSPHATE SYNTHASE FROM MULBERRY LEAVES: STEREOCHEMISTRY OF HYDROGEN ELIMINATION IN THE PRENYLTRANSFERASE REACTION

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Key Word Index—*Morus bombysis*; Moraceae; mulberry; stereochemistry; prenyltransferase; polyprenyl diphosphate synthase; isopentenyl diphosphate; hydrogen elimination.

Abstract—A polyprenyltransferase catalysing the formation of *Z*-double bonds was partially purified from mulberry leaves, *Morus bombysis*. The enzyme catalysed a consecutive condensation of isopentenyl diphosphate with geranylgeranyl diphosphate as an allylic primer to produce a series of ficaprenol-type *Z,E*-mixed polyprenyl diphosphates with carbon chain length ranging from C_{40} to C_{60}. Not only *all-E*-geranylgeranyl diphosphate but also *E,E*-farnesyl- and geranyl diphosphates were accepted as substrates. Addition of Triton X-100 stimulated the enzymatic activity. The stereochemistry of hydrogen elimination from the 2-position of isopentenyl diphosphate during the *Z*-prenyl chain formation was examined directly by experiments using this synthase and (*S*)-[1-^{14}C, 2-^{3}H]isopentenyl diphosphate and it was demonstrated that the 2-pro-*S* hydrogen was lost. Feeding experiments of stereospecifically ^{3}H-labelled mevalonic acid with intact mulberry leaves, however, showed that mevalonic acid was incorporated into polyprenols with elimination of the 4-pro-*S* hydrogen of the acid.

INTRODUCTION

It was once generally accepted that the 4-pro-*S* hydrogen of mevalonic acid (MVA), which is equivalent to the pro-*R* hydrogen at C-2 of isopentenyl diphosphate (IPP), is lost in the formation of *E*-isoprene residues, whereas the 4-pro-*R* hydrogen (equivalent to the 2-pro-*S* hydrogen of IPP) is lost in the formation of *Z*-isoprene residues [1–3].

Hydrogen elimination in the formation of *Z*-isoprene residues was first studied in the biosynthesis of rubber by Archer *et al.* [2], who demonstrated that the 4-pro-*R* hydrogen of MVA was eliminated during the *Z*-isoprene residue formation. Examples of 4-pro-*R* hydrogen elimination were also reported in the biosynthesis of the *Z*-prenyl portions of hexahydroprenol in the mycelium of *Aspergillus fumigatus* [4] and of betulaprenols in the woody tissue of *Betula verrucosa* [5]. 4-Pro-*R* hydrogen elimination was also reported in the biosynthesis of the *Z*-isoprene residues of dolichols in rat liver [6]. *In vitro* experiments with partially purified prenyltransferases from *Lactobacillus plantarum* [7], *Micrococcus luteus* [8], *Bacillus subtilis* [9] and *Paracoccus denitrificans* [10] have directly demonstrated that the 2-pro-*S* hydrogen of IPP is eliminated during the enzyme reactions that form *Z*-prenyl chains. Recently, however, some papers have appeared reporting that *Z*-prenyl residues are formed in some higher plants with retention of the 4-pro-*R* hydrogen of MVA [11–15].

In order to refine stereochemical aspects of the prenyltransferase reaction that forms *Z*-prenyl residues, it is now necessary to obtain direct evidence for hydrogen elimination by using enzymes from higher plants. This paper describes the stereospecific removal of the 2-pro-*S* hydrogen of IPP during its incorporation into the *Z*-prenyl moiety of polyprenyl diphosphate with *Z,E*-mixed prenyl residues by the action of a prenyltransferase extracted from mulberry leaves, *Morus bombysis*.

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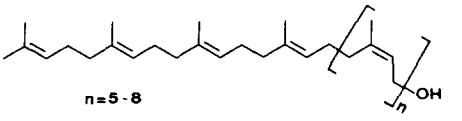
RESULTS

Detection of polyprenyl diphosphate synthase in the cell-free extract of mulberry leaves

It has been reported that mulberry leaves (*M. bombysis*) contain ficaprenol type polyprenols that consist of one dimethylallyl terminal unit (ω-terminal), three *E*-isoprene residues and a sequence of five to eight *Z*-isoprene residues aligned in that order [16]. In order to



obtain a cell-free system which contained prenyltransferase catalysing the formation of polyprenyl diphosphate with *Z*-prenyl residues of this type from IPP and an allylic diphosphate, we prepared a crude extract of mulberry leaves. As shown in Table 1, significant prenyltransferase activity was detected in the 108 000 *g* supernatant of the extract when allylic diphosphates were added as priming substrates. Moreover, the enzymatic activity was stimulated by Triton X-100, suggesting that the supernatant contained polyprenyl diphosphate synthase which, like undecaprenyl diphosphate synthase from bacteria [7–9], could be activated by detergent.

Table 1. Prenyltransferase activities in the 108 000 *g* supernatant of mulberry leaf extract

Allylic substrate	Activity/dpm *	Activity/dpm †
None	662	793
DMAPP	4174	12741
GPP	10182	18986
E,E-FPP	6216	13716
E,E,E-GGPP	2287	11914
Z,E,E-GGPP	977	11309

The enzymatic activities were assayed with [1-^{14}C]IPP and various allylic diphosphates in the presence or absence of Triton X-100 as described in *Experimental*.

* Assayed in the absence of Triton X-100.

† Assayed in the presence of Triton X-100.

Product analysis

The *n*-BuOH extracts from the reaction mixture of [1-^{14}C]IPP and *E,E*-farnesyl diphosphate (FPP) in the presence of Triton X-100 were analysed by radio-TLC. The radioactivity of the product was detected as a peak (R_f value, 0.46) between those of *all-E*-geranylgeranyl diphosphate (GGPP) and *all-E*-geranylgeranyl monophosphate, indicating that the radioactive product was polyprenyl diphosphate(s) with carbon chain-length longer than C_{20}.

The radio TLC chromatograms of the hydrolysate resulting from incubations of [1-^{14}C]IPP and geranyl diphosphate (GPP), FPP or GGPP in the presence of Triton X-100 showed a broad radioactivity peak over the R_f range of 0.6–0.8 in every case of incubation, indicating that the supernatant contained polyprenyl diphosphate synthase(s). For further analysis the polyprenols obtained by the reaction of [1-^{14}C]IPP and GGPP were subjected to radio-HPLC analysis. Five radioactivity peaks were observed at retention times of 9.5, 12.7, 16.4, 22.2, and 29.6 min. The major peak appeared at 16.4 min, which was slightly longer than that of *all-E*-decaprenol (15 min). The major polyprenol fraction obtained by a large-scale incubation was purified by HPLC and subjected to mass spectrometry. The polyprenol exhibited typical fragmentation patterns of an isoprenoid alcohol, showing an $[M]^+$ at *m/z* 698 ($C_{50}H_{82}O$) with fragment ions at *m/z* 680, 611, 543, 475, 407, 339, 271, 203, 135, and 69 (base peak). The mass spectrum and the chromatographic mobility described above indicate that the major product is decaprenyl diphosphate with *Z,E*-mixed stereochemistry of the ficaprenol type. Thus, it is reasonable to assume that the products synthesized by the incubation of IPP and GGPP in the presence of Triton X-100 are a series of similar *Z,E*-mixed type polyprenyl diphosphates with carbon chain lengths ranging from C_{40} to C_{60}. Even when GGPP was substituted with GPP or FPP, the polyprenol products gave radio-HPLC chromatograms similar to that obtained from GGPP. Hence, the polyprenyl diphosphate synthase activity found in the 108 000 *g* supernatant is responsible for the synthesis of the polyprenols found in mulberry leaves [16].

On the other hand, the enzymatic reaction product obtained by the incubation of [1-^{14}C]IPP and GPP in the absence of Triton X-100 was assigned as FPP, which was identified as *E,E*-farnesol after phosphatase treatment [17] followed by radio-TLC analysis (data not shown). This result indicates that FPP synthase is also extracted in the supernatant of mulberry leaf homogenate.

Seasonal variation in the level of prenyltransferase(s)

It has been reported that with increasing age of leaves there is a corresponding rise in the content of polyprenyl products in some higher plants [18, 19]. We examined the concentrations of prenyltransferases detectable in the 108 000 *g* supernatant of mulberry leaf homogenates. With maturing of the leaves a gradual increase in the amount of polyprenyl diphosphate synthase activity was observed (Fig. 1). On the contrary, FPP synthase activity decreased markedly in early summer. We therefore used mulberry leaves harvested in the middle of September as enzyme source.

Partial purification of polyprenyl diphosphate synthase

Because ammonium sulphate fractionation of the 108 000 *g* supernatant, which was viscous, resulted in a marked loss of the prenyltransferase activity, the supernatant was directly chromatographed on a DEAE-Toyopearl column. A fraction which showed prenyltransferase activity in the presence of Triton X-100 was obtained with *ca* 8% recovery of enzymatic activity (Fig. 2). Product analysis of the enzymatic reaction, however, showed that the fraction was still contaminated with FPP synthase. Further purification by hydroxylapatite or Sephadex G-100 chromatography was not successful.

Stereochemistry of hydrogen elimination at 2-position of IPP

The stereochemical course of the polyprenyl diphosphate synthase reaction was determined directly using the DEAE-Toyopearl purified enzyme or the 108 000 *g* supernatant with (*S*)-[1-^{14}C, 2-^{3}H]IPP and GGPP as substrates. Since pig liver FPP synthase is known to

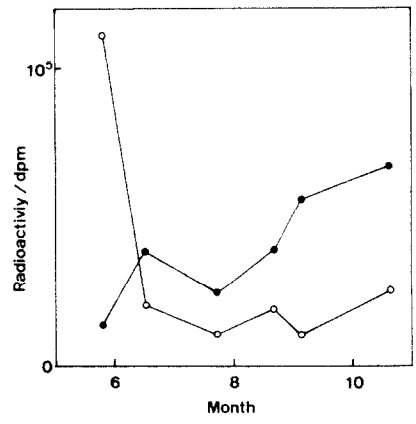


Fig. 1. Seasonal variation of the concentrations of prenyltransferases in mulberry leaves. ●, polyprenyl diphosphate synthase activity; ○, FPP synthase activity.