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ISOPENTENYL DIPHOSPHATE ISOMERASE ACTIVITY IN *LACTARIUS* MUSHROOMS

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Key Word Index—*Lactarius* sp.; mushroom; natural rubber; biosynthesis; polyisoprene; isopentenyl diphosphate; isopentenyl diphosphate isomerase.

Abstract—Isopentenyl diphosphate (IDP) isomerase [EC 5.3.3.2] activity, which is presumed to be involved in rubber biosynthesis, was detected in a homogenate of *Lactarius* mushrooms by measuring the radioactive C_5 alcohols derived from [1-^{14}C] IDP. Fluoride ions were added to the assay mixture to inhibit phosphatase activity, which interfered with the isomerase activity by hydrolysing the IDP substrate. The level of formation of [1-^{14}C] DMADP in the homogenate of *L. volemus* was 41%. © 1997 Elsevier Science Ltd

INTRODUCTION

Isopentenyl diphosphate (IDP) isomerase and prenyltransferases have been isolated from various organisms [1–4]. In the initiating step of the biosynthesis of *trans*-isoprenoid compounds, IDP isomerase catalyses the formation of DMADP, which acts as a primer to start the successive addition of isopentenyl units from IDP to build up polyprenyl diphosphates of a required chain length.

Biochemical studies in the 1960s assumed that rubber formation starts with the addition of IDP to DMADP and subsequent chain elongation by successive addition of IDP as occurs in the biosynthesis of terpenoids. The presence of IDP isomerase in *Hevea* latex was suggested. Ozonolytic degradation of GGDP formed by incubation of *Hevea* latex with [4-^{14}C] IDP gave ^{14}C-acetone in agreement with the formation of ^{14}C-GGDP *via* ^{14}C-DMADP [5]. This is strong evidence for the presence of IDP isomerase in *Hevea* latex. Recently, the stimulating effect of various allylic diphosphates has been examined by co-incubation of ^{14}C-IDP with washed rubber particles to clarify the initiating species of rubber formation. It has been confirmed that C_5 to C_{20} allylic diphosphates have a stimulatory effect on rubber formation in a system containing IDP and washed rubber particles from *H. brasiliensis* [5–7]. Direct incorporation of [1-^{3}H]neryl diphosphate and [1-^{3}H]geranyl diphosphate was reported [8]. The efficiency of the stimulatory effect increased slightly with the chain length of the allylic diphosphate from C_5 to C_{20}, rather than with the geometric isomerism of isoprene units [5, 8]. These findings suggested that the direct initiator is FDP and/or GGDP rather than DMADP in the case of *H. brasiliensis*.

^{13}C NMR studies showed the presence of two to three *trans* isoprene units linked to the dimethylallyl group at the terminal end in rubbers from leaves of Goldenrod (*Solidago altissima*) and Sunflower (*Helianthus annuus*) [9–11]. However, *Hevea* rubber showed no signal due to the putative terminal dimethylallyl group, although ^{13}C NMR signals corresponding to the *trans*-isoprene units in the *trans*, *trans*, *cis*-sequence were detected [11, 12]. Therefore, no direct evidence has been given from the structural information for the presence of IDP isomerase in *Hevea* latex.

NMR spectroscopic studies demonstrated the presence of the dimethylallyl-*trans*, *trans*-sequence in the rubber from sporophores of *Lactarius* mushrooms, showing that the initiating species of the rubber formation is *trans*, *trans*-FDP [13, 14]. In this paper, we have shown the presence of IDP isomerase in sporophores of *Lactarius* mushrooms producing rubber as latex.

RESULTS AND DISCUSSION

To detect IDP isomerase activity in the homogenate of *L. volemus*, we supplemented the assay mixture with enough fluoride ion to inhibit the phosphatase activity in the homogenate, which would interfere with the

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Table 1. Radioactivities of enzymatic reaction products formed on incubation for 1 hr of a homogenate of *L. volemus* sporophores with ^{14}C-IDP (1.06×10^5 dpm) (see Experimental)

Stepwise extraction solvents	Radioactivity of extract (dpm)	Major enzymatic activity
1. Diethyl ether (−KF)	13,500±430	phosphatase
2. Diethyl ether (+KF)	2,190±260	phosphatase(inhibited)
3. 1-Butanol (+KF, −FDP)	720±93	prenyltransferase
4. 1-Butanol (+KF, +FDP)	3,050±570	prenyltransferase
5. Diethyl ether (+KF, −FDP)	36,810±1580	IDP isomerase

The values shown are means±S.E. for three experiments.

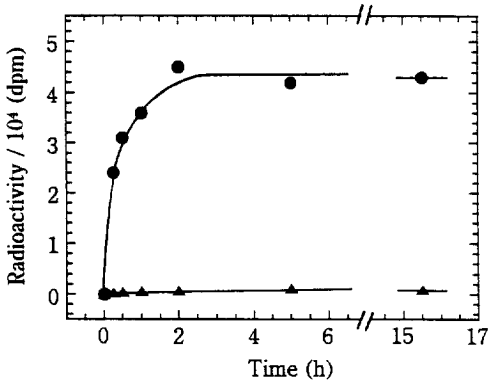

Fig. 1. IDP isomerase activity in a *L. volemus* homogenate. The enzymatic activity was measured as described in Experimental (^{14}C-IDP, 1.06×10^5 dpm). ●, homogenate; ▲, homogenate boiled for 1 hr.

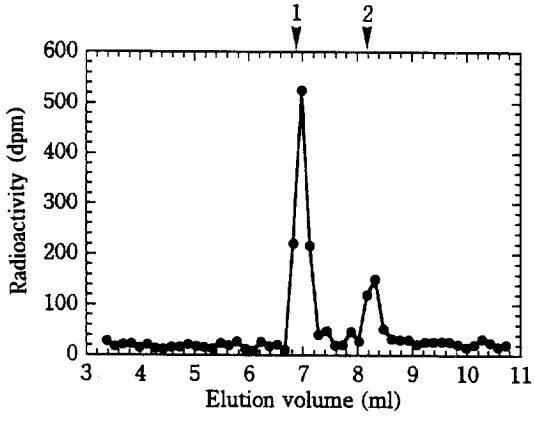

Fig. 2. HPLC analysis of the materials produced on acid-treatment of the product formed from ^{14}C-IDP, which remains after the purification steps described in Experimental. Arrows indicate the retention volumes of authentic dimethylvinyl carbinol (**1**) and dimethylallyl alcohol (**2**), respectively.

isomerase activity by hydrolysing the IDP substrate. It was found that more than 80% of the phosphatase activity was inhibited by the addition of 100 mM KF (Table 1, c.f. lines 1 and 2). The low radioactivity content of the butanol extract (Table 1, line 3) indicated that a small amount of polyprenyl diphosphate(s) was synthesized in the incubation without FDP. In the presence of IDP and FDP (line 4), a larger amount of radioactivity was found in the butanol extract, indicating the presence of a prenyltransferase employing FDP as the allylic primer. A large amount of radioactive material was detected in the ether extract of the hydrolysate of the residues remaining after butanol extraction (line 5). This was attributable to the DMADP produced as a result of IPP isomerase activity. The conversion of IDP to acid-labile DMADP increased with time of incubation up to 2 hr and attained a final level of 41% (Fig. 1).

The radioactivity of the run-through fractions of HPLC of the products obtained from the incubation of ^{14}C-IDP and the mushroom homogenate (Fig. 2). Two peaks were clearly observed. The larger radioactivity peak agreed well with the elution volume of dimethylvinyl carbinol. The elution volume of another peak corresponded to that of dimethylallyl alcohol. It is well known that DMADP is readily hydrolysed with acid to yield a mixture of the two isomeric alcohols and that the formation of the tertiary alcohol is predominant [15]. These observations clearly indicate that the radioactivities shown in line 5 of Table 1 and in Fig. 1 represented the sum of dimethylvinyl carbinol and dimethylallyl alcohol which were formed *via* ^{14}C-DMADP.

The presence of IDP isomerase and prenyltransferase was confirmed in the homogenate of *L. volemus* sporophores. The activity of prenyltransferase was lower than that of IDP isomerase under the condition of incubation. Since the level of phosphatase activity was high in the homogenate of *L. volemus* sporophores, it is necessary to estimate the actual activity of IDP isomerase by inhibiting the activity of phosphatase. IDP isomerase exhibits a pH optimum of 7.5 and optimum temperature around 25–30°. A divalent cation (Mg^{2+}, >2 mM) is required for maximum enzymic activity. These characteristics are quite similar to those of IDP isomerase from *Hevea* latex [16].

IDP isomerase activity was detected in the homogenates of four other (*L. gracilis*, *L. insulsus*, *L. quietus* and *L. torminusus*) *Lactarius* species (Table 2). Rubber was isolated from sporophores of *L. gracilis* and *L. insulsus*, but the isolation from the other two was unsuccessful because of the insufficient amounts of sporophores. The isomerization of ^{14}C-IDP in the homogenates of *Lactarius* mushrooms reached

Table 2. The activity of IDP isomerase in *Lactarius* mushrooms

Sample	Radioactivity (dpm)
L. volemus	48,400 ± 1750
L. gracilis	53,000 ± 3430
L. insulsus	63,200 ± 3300
L. quietus	28,400 ± 2680
L. torminusus	17,200 ± 980

The enzyme activity was measured as described in the Experimental, except for the use of a 5 hr incubation time (^{14}C-IDP, 1.06×10^5 dpm).

The values shown are means ± S.E. for three experiments.

remarkably high levels, ranging from 16 to 58%. On the other hand, ozonolysis of rubber obtained by incubating *Hevea* latex with [4-^{14}C] IDP gave no ^{14}C-acetone which is expected to be derived from the dimethylallyl group [17]. This was attributed to the extremely small mass fraction of the terminal group in comparison with the internal isoprene units. The number average molecular weight ($1–2 \times 10^4$) of *L. volemus* rubber [13] is much lower than that ($2–5 \times 10^5$) of *Hevea* rubber. The M_r of natural polyisoprenes may be determined by the ratio of IDP units to the initiating species, FDP or GGDP.

This study showed the presence of IDP isomerase needed for the initiation of the biosynthesis of rubber.

EXPERIMENTAL

Materials. Sporophores of *Lactarius* mushrooms were collected in Fukushima, Tochigi and Miyagi, Japan in August to October, 1995. Fresh sporophores (30 g) were cut into small pieces, homogenized in a Waring Blender and suspended in 60 ml of 50 mM Tris–HCl buffer, pH 7.5, containing 5 mM $MgCl_2$. This suspension was used for all of the assays reported in this paper. [1-^{14}C] IDP (52 mCi mmol^{-1}) was purchased from Amersham. Unlabelled FDP was synthesized according to the procedure of reference [18]. 2-Mercaptoethanol was a product of Merck. Dimethylallyl alcohol and dimethylvinyl carbinol were obtained from Tokyo Chemical Industry (Tokyo, Japan). All other reagents were of analytical reagent grade.

Incubation conditions and stepwise extraction of enzymic products. The incubation mixt. for the enzymatic analysis contained, in a final vol. of 1 ml, 50 mM Tris–HCl buffer, pH 7.5, 5 mM $MgCl_2$, 100 mM KF, 50 mM 2-mercaptoethanol, 0.92 μM [1-^{14}C]IDP (52 mCi mmol^{-1}; 1.06×10^5 dpm per incubation) and 650 μl of the mushroom homogenate. The incubation was carried out at 27° for 1 hr, and then stepwise extraction was performed as follows. The reaction was stopped by chilling the reaction mixt. in an ice bath. After addition of 2 ml H_2O satd with NaCl, the mixt. was treated with 3.0 ml Et_2O and the radioactivity in the organic layer was counted in a liquid scintillation counter (Aloka, type LSC-1000) to estimate phosphatase activity. The aq. layer was washed thoroughly with Et_2O to remove remaining phosphatase products, and then extracted with 3 ml 1-BuOH, which had been satd with H_2O, to estimate the activity of prenyltransferases forming oligoprenols with chain-length $>C_{15}$. After thorough washing with 1-BuOH, the aq. layer was acidified with 500 μl 1 M HCl followed by incubation at 37° for 15 min to complete the hydrolysis of acid-labile DMADP formed. The mixt. was treated with Et_2O and the radioactivity in the ethereal layer was counted to determine the amount of DMADP produced.

IDP isomerase assay. The incubation was performed at 27° for 15 min to 15.5 hr, and then the reaction was stopped by chilling in an ice bath. After addition of 2 ml H_2O satd with NaCl, the mixt. was extracted with Et_2O (3 ml × 3) to remove alcohols produced by the action of phosphatases and then extracted with 1-BuOH (3 ml × 3) to remove the prenyl diphosphates ($>C_{15}$) synthesized by prenyltransferases. The hydrosis of acid-labile DMADP was carried out by incubation at 37° for 15 min with 500 μl 1 M HCl. The mixt. was treated with Et_2O and the radioactivity in the ethereal layer was counted.

Prenyltransferase assay. The assay mixt. was similar to that for IDP isomerase assay as described above except that 2.5 μM FDP was added. After incubation at 27° for 1 hr, the reaction was stopped by chilling the mixt. in an ice bath. Then the mixt. was extracted with Et_2O thoroughly and the products were extracted with 3 ml 1-BuOH which had been satd with H_2O. The radioactivity in the BuOH extracts was counted.

Identification of enzymatic products by HPLC. The HPLC measurements were performed on a silica-gel column (Du Pont, 4.6 mm int. diameter × 250 mm) using hexane–2-PrOH (5:1) as eluent. The terpene alcohols, dimethylvinyl carbinol and dimethylallyl alcohol were used to calibrate the elution. Frs (0.15 ml) were collected at a flow rate of 0.3 ml min^{-1} and their radioactivity was determined.

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