Pergamon

Phytochemistry, Vol. 43, No. 4, pp. 769–772, 1996

0031-9422/96 $15.00 + 0.00

PII: S0031-9422(96)00374-3

ISOPENTENYL DIPHOSPHATE ISOMERASE IN RUBBER LATEX

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(*Received in revised form* 16 *May* 1996)

Key Word Index—*Hevea brasiliensis*; Euphorbiaceae; rubber latex; isopentenyl diphosphate isomerase; rubber biosynthesis.

Abstract—Isopentenyl diphosphate isomerase (EC 5.3.3.2), which catalyses the reversible isomerization of isopentenyl diphosphate to dimethylallyl diphosphate, is presumed to be involved in rubber biosynthesis, but no direct evidence has been given for its occurrence in rubber latex. This enzyme activity was found in the C-serum powder prepared by lyophilization of centrifuged rubber latex of *Hevea brasiliensis*. Characterization of partially purified enzyme showed a K_m value of 71 μM for isopentenyl diphosphate, a pH optimum of 7 and an optimum temperature at 37°. Divalent cations (Mg^{2+} or Mn^{2+}) and dithiothreitol are required for maximum enzymic activity. Sulphhydryl reagents such as iodoacetamide, *p*-chloromercuribenzoate and *N*-ethylmaleimide are effective inhibitors. Copyright © 1996 Published by Elsevier Science Ltd

INTRODUCTION

Isopentenyl diphosphate (IDP) isomerase (EC 5.3.3.2) catalyses the reversible isomerization of IDP to dimethylallyl diphosphate (DMADP). This enzyme provides the primer allylic substrate DMADP, for the initial condensation of isoprene units in the biosynthesis of isoprenoid compounds [1]. As reviewed by Audley and Archer [2], IDP isomerase has been presumed to be present in *Hevea* latex for the initiation of rubber biosynthesis. The presence of IDP isomerase in *Hevea* latex was suggested first by the fact that the amount of incorporation of [2-^{14}C]mevalonate into non-rubber lipids was reduced markedly in the presence of iodoacetamide, a potent inhibitor of the isomerase [3]. Further evidence is that ozonolysis of the geranylgeraniol derived from the incubation of *Hevea* latex with [4-^{14}C]IDP resulted in the formation of ^{14}C-labelled acetone, which indicates the direct conversion of IDP into the allylic primer substrate for geranylgeranyl diphosphate synthesis [4]. Recently, Cornish has reported an indirect proof for the presence of the enzyme in latex by using a specific inhibitor [5]. This paper describes direct evidence for the presence of the enzyme in *Hevea* latex, and its characterization.

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RESULTS AND DISCUSSION

Detection of IDP isomerase activity in rubber latex

This was carried out using freshly lyophilized C-serum [6]. Sufficient fluoride was added to inhibit phosphatases, which would interfere in the assay. As shown in Table 1 (column 2), more than 90% of the

Table 1. Stepwise extraction of enzymic reaction productions formed on incubation of [1-^{14}C]IDP with C-serum. Free alcohols formed by phosphatases were extracted with ether (1 and 1A), then polyprenyl diphosphates (>C_{15}) with H_2O saturated 1-butanol (2). DMADP remaining was hydrolysed with HCl, and the products extracted into ether (3) (see Experimental)

Extraction step/ solvent		Radioactivity* in extract (dpm)	Major enzymic activity detected
1	Et_2O*	11 750	Phosphatases
1A	Et_2O†	860	Phosphatases (inhibited)
2	1-BuOH	11 390	Prenyltransferases
3	Et_2O	13 910	IDP isomerase

*Each value represents the average of three determinations.
†1A was used for subsequent operations.

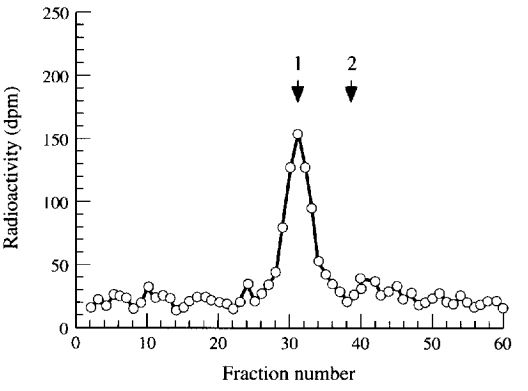

Fig. 1. HPLC analysis on a silica gel column of the [^{14}C]-labelled ether-soluble acid hydrolysis products of DMADP. Arrows indicate the retention volumes of authentic dimethylvinyl carbinol (1) and dimethylallyl alcohol (2), respectively.

phosphatase activity was inhibited by 100 mM KF. Detection of a large amount of radioactivity in the butanol extracts indicates the presence of prenyltransferase(s), which produce prenyl diphosphates longer than C_{15} [2]. Moreover, a significant amount of radioactivity of the product of IDP isomerase was detected in the ether extracts of the hydrolysate of the residue after the butanol extraction (Table 1). HPLC analysis (Fig. 1) demonstrates that the radioactive allylic diphosphate remaining in the reaction mixture after thorough extraction with butanol is DMADP, which is readily hydrolysed with acid to yield the expected mixture of dimethylvinyl carbinol and dimethylallyl alcohol. Other radioactivity peaks corresponding to the tertiary and primary alcohols derived from C_{10}- and C_{15}-prenyl diphosphates were not detected at all, indicating that all prenyl diphosphates ($>C_{15}$) formed by the action of prenyltransferases were extracted completely, and that geranyl diphosphate (GDP) was not formed. These findings suggest the presence of IDP isomerase in the lyophilized C-serum of *Hevea* latex.

DEAE–Toyopearl chromatography of C-serum

The presence of the isomerase in C-serum was confirmed by fractionation of a DEAE–Toyopearl column. The enzyme eluted after a large peak of prenyltransferase (Fig. 2). Incubation of fractions 29–31 (Fig. 2) with [1-^{14}C]IDP under conditions used to assay the isomerase, followed by HPLC analysis, gave a similar chromatogram to that of Fig. 1, indicating that DMADP was the only product (data not shown).

Characteristics of the IDP isomerase

The enzyme requires divalent cations for its activity (Fig. 3). Divalent Mn is the most effective, at around 0.1 mM, but is inhibitory at higher concentrations. Divalent Mg, Zn^{2+} and Co^{2+} are also activators, but Zn^{2+} and Co^{2+} are inhibitory at higher concentrations.

Figure 4 shows that the isomerase is highly sensitive to sulphhydryl reagents such as iodoacetamide (IAA), *N*-ethylmaleimide (NEM) and *p*-chloromercuribenzoate (PCMB), and the addition of more than 1 mM dithiothreitol (DTT) is required for maximum activity. Thus an SH group(s) is essential for catalytic activity.

The enzyme exhibits a rather broad pH optimum between 7 and 8 with maximum activity around pH 7 (data not shown). The K_m value at pH 7 for IDP was calculated as 71 μM from the Lineweaver–Burk plot (data not shown). The optimum temperature is 37°.

These characteristics are quite similar to those of IDP isomerases from other organisms [7, 8].

The above findings support the earlier work, and

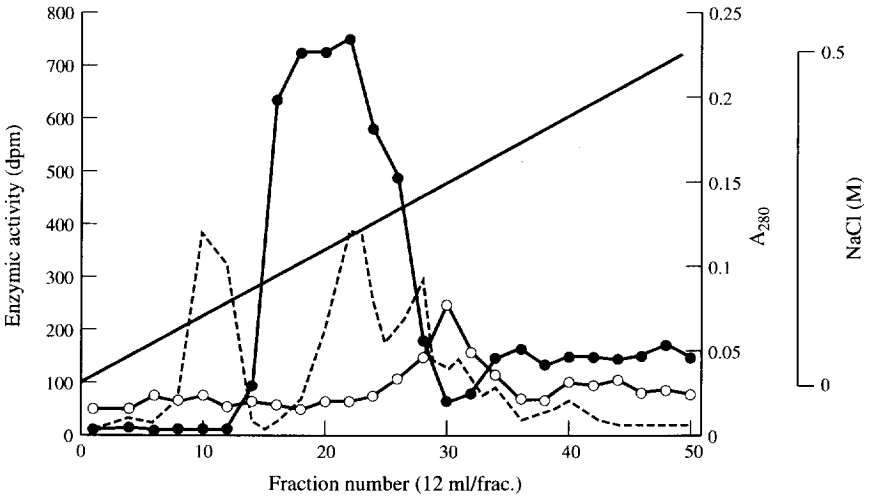

Fig. 2. DEAE–Toyopearl chromatography of C-serum: ○, IDP isomerase activity; ●, prenyltransferase activity; ----, absorbance at 280 nm; ——, concentration of KCl.

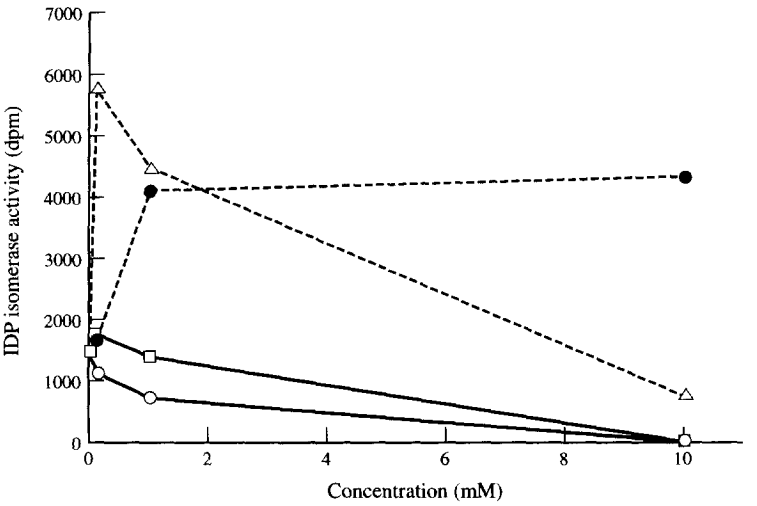

Fig. 3. Effect of concentration of divalent metal ions on IDP isomerase activity: ●, $MgCl_2$; △, $MnCl_2$; □, $CoCl_2$; ○, $ZnCl_2$.

provide clear evidence that IDP isomerase is a component of *Hevea* latex. However, it must be emphasized that the role of the enzyme in rubber formation in the species remains to be elucidated, because NMR analysis of *Hevea* rubber failed to indicate the presence of the expected ω-dimethylallyl group [9].

EXPERIMENTAL

Chemicals. [1-^{14}C]IDP (1.924 GBq mmol^{-1}) was purchased from Amersham and was diluted with non-labelled IDP before use. Unlabelled IDP, DMADP and GDP were synthesized according to the procedure of ref. [10] DTT was a product of Seikagaku Kogyo (Tokyo, Japan). IAA and PCMB were purchased from Nacalai Tesque (Kyoto, Japan). 2-Mercaptoethanol was a product of Merck. DEAE–Toyopearl was purchased from Tosoh (Tokyo, Japan). NEM, dimethylallyl alcohol and dimethylvinyl carbinol were obtained from Tokyo Chemical Industry (Tokyo, Japan). All other reagents were of analyt. reagent grade.

Preparation of Hevea *C-serum powder.* The latex was obtained from several regularly tapped (half-spiral cut, every other day) high-yielding trees (*H. brasiliensis*, clone RRIM 600), each *ca* 17 years old. After tapping, the latex was collected for 50 min into an ice-chilled beaker and then centrifuged at 49 000 *g* for 40 min to give the C-serum [6], which was immediately lyophilized.

Incubation conditions and stepwise extraction of enzymic products. The incubation mixt. contained, in a final vol. of 1 ml, 100 mM $MgCl_2$, 200 mM KF, 100 mM 2-mercaptoethanol, 1.84 μM [1-^{14}C]IDP (1.924 GBq mmol^{-1}), 5 μM GDP and 30 mg C-serum powder dissolved in 300 μl of 1 M K-Pi buffer, pH 7.0. Incubation was carried out at 30° overnight, and then stepwise extraction was performed as follows. After

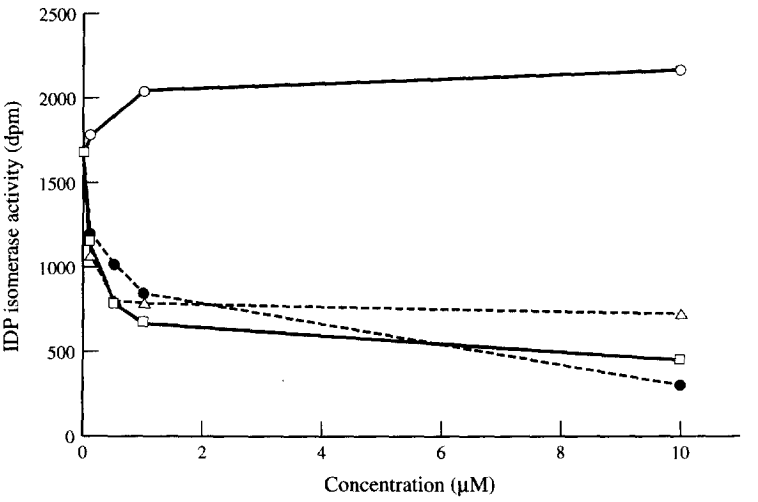

Fig. 4. Effect of SH-reagents and DTT on IDP isomerase activity: ●, IAA; □, NEM; △, PCMB; ○, DTT.

addition of 2 ml satd NaCl soln, the mixt. was treated with 3.0 ml Et_2O and the radioactivity in 1.0 ml of the organic layer counted in a liquid scintillation counter (Aloka, type LSC-1000) to estimate phosphatase activity. The aq. layer was washed with Et_2O to remove remaining phosphatase products, and then extracted with 3.0 ml H_2O-satd 1-BuOH to estimate prenyltransferase activity ($>C_{15}$). After thorough washing with BuOH, the aq. layer was acidified with 100 μl 6 M HCl and incubated at 37° for 15 min. The mixt. was again extracted with 3.0 ml Et_2O, and the radioactivity in 1.0 ml of the Et_2O layer counted to determine the amount of DMADP produced.

DEAE–Toyopearl chromatography. All steps were carried out at 4°. 200 mg C-serum powder in 25 mM Tris–HCl buffer, pH 8.5, were applied to a DEAE–Toyopearl column (3.5 × 18 cm) equilibrated with 25 mM Tris–HCl buffer, pH 8.5, containing 1 mM EDTA and 10 mM 2-mercaptoethanol. Elution was performed with a linear gradient of 0–500 mM NaCl in the same buffer.

IDP isomerase assay. The assay mixt. contained, in a final vol. of 0.5 ml, 10 mM $MgCl_2$, 0.1 mM $MnCl_2$, 10 mM DTT, 50 mM KF, 50 μM [1-^{14}C]IDP (37 GBq mol^{-1}), 100 mM Tris–HCl buffer, pH 7.0, and the enzyme fr. (50 μg protein of the partially purified enzyme). The mixt. was incubated at 30° for 1 hr, and the reaction stopped by chilling in an ice bath. After addition of 1 ml satd NaCl soln, the mixt. was extracted with 3× 3 ml portions of Et_2O, followed by extraction with 3× 3 ml portions of H_2O-satd 1-BuOH to remove alcohols produced by the action of phosphatases, and the prenyl diphosphates ($>C_{15}$) synthesized by prenyltransferases, respectively, and then acidified with 200 μl 6 M HCl, followed by incubation at 37° for 15 min. The mixt. was treated with 3.0 ml Et_2O and the radioactivity in 1.0 ml of the Et_2O layer counted.

Small amounts of authentic dimethylvinyl carbinol and dimethylallyl alcohol (each a few mg) were then added to the Et_2O layer as carriers, the solvent removed by evapn in a water bath, and the radioactive products analysed by HPLC on a silica gel column (Du Pont Instruments, 4.6 × 250 mm). Hexane–2-PrOH (20:1) was used as eluent. Frs (0.15 ml) were collected at 0.3 ml min^{-1}, and the radioactivity in each fr. determined. Protein concn was estimated using Protein Assay (Bio-Rad Laboratories) with BSA as the standard.

Prenyltransferase assay. The assay mixture was that used for IDP isomerase, except that 5 μM GDP was added. After incubation at 30° for 1 hr, the reaction was stopped by chilling as above. The mixture was then washed with Et_2O thoroughly, the aq phase extracted with 3.0 ml of water sat. 1-BuOH and the radioactivity in 1.0 ml of the BuOH extract counted.

Acknowledgements—This work was supported by the Monbusho International Scientific Research Program 5044029 and by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Poulter, C. D. and Rilling, H. C. (1981) in *Biosynthesis of Isoprenoid Compounds* (Spurgeon, S. L. and Porter J. W., eds), Vol. 1, pp. 161–224. John Wiley, New York.
2. Audley, B. G. and Archer, B. L. (1988) in *Natural Rubber Science and Technology* (Roberts, A. D., ed.), pp. 35–62. Oxford University Press, London.
3. Chesterton, C. J. and Kekwick, R. G. O. (1966) *Biochem. J.*, **100**, 56.
4. Archer, B. L. and Audley, B. G. (1986) in *Biochemistry and Regulation of* cis-*Polyisoprene in Plants* (Benedict, C. R., ed.), pp. 235–252. Proceedings of NSF Sponsored Workshops held at Texas A & M University.
5. Cornish, K. (1993) *Eur. J. Biochem.* **218**, 267.
6. Wititsuwannakul, R., Wititsuwannakul, D. and Dumkong, S. (1990) *Phytochemistry* **29**, 1755.
7. *Enzyme Handbook 2* (1990) Springer-Verlag, Berlin, Heidelberg.
8. Ogura, K., Nishino, T., Koyama, T. and Seto, S. (1971) *Phytochemistry* **10**, 779.
9. Tanaka, Y. (1989) *J. Appl. Polyn. Sci., Appl. Polm. Symp.*, **44**, 1.
10. Davisson, V. J., Woodside, A. B., Neal, T. R., Stremler, K. E., Muehlbacher, M. and Poulter, C. D. (1986) *J. Org. Chem.* **51**, 4768.