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HYDROXYMETHYLGLUTARYL CoA REDUCTASE (NADPH) IN THE LATEX OF *HEVEA BRASILIENSIS*

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Key Word Index—*Hevea brasiliensis*; Euphorbiaceae; latex; lutoids; hydroxymethylglutaryl CoA reductase; ATP-inactivation.

Abstract—The activity of hydroxymethylglutaryl CoA reductase (NADPH) (EC 1.1.1.34) was studied in the latex of regularly tapped mature trees of *Hevea brasiliensis*. The reductase activity was found mainly (95% of the total activity) in the pellet fraction (40 000 *g*) of the centrifuged latex. The enzyme in this fraction had a specific requirement for NADPH as the cofactor and, while not obligatory for activity, was activated by dithiothreitol at the optimum concentration of 2 mM. The pH optimum was found to be 6.6–6.9 in 0.1 M phosphate buffer. Mevalonate and CoA (at 2 mM each) did not affect enzyme activity, while hydroxymethylglutarate (2 mM) was slightly inhibitory. *p*-Chloromercuribenzoate (1 mM) completely inhibited this enzyme. The reductase activity in the 40 000 *g* pellet was not easily solubilized either using Triton X-100 or by sonication. The apparent K_m for the washed, membrane-bound enzyme (103 000 *g* pellet) was 56 μM (*RS*-HMG-CoA). Magnesium-ATP (4 mM) inactivated the reductase but this effect was greatly diminished or was absent upon washing the 40 000 *g* pellet.

INTRODUCTION

Natural rubber from *Hevea brasiliensis* is a *cis*-polyisoprenoid compound of high MW (10^5–4×10^6) derived from isopentenyl pyrophosphate. The latter compound is also the common precursor for many other isoprenoids, such as carotenes and sterols [1]. One of the reactions in the biosynthesis of isopentenyl pyrophosphate from acetate is the NADPH-linked reduction of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) to mevalonate, catalysed by the enzyme hydroxymethylglutaryl CoA reductase (NADPH) (HMG-CoA reductase; EC 1.1.1.34). The occurrence of this enzyme in the latex of *H. brasiliensis* has been reported by Hepper and Audley in their study of the incorporation of labelled HMG-CoA into rubber [2]. Their study of the reductase, however, was limited to the observation that the enzyme was particulate (600 *g* pellet) and that it was unstable in the absence of a thiol. Moreover, these workers obtained their latex from seedlings of *H. brasiliensis* grown under glasshouse conditions. This paper reports on the occurrence and some of the properties of HMG-CoA reductase from the latex obtained from regularly tapped mature trees of *H. brasiliensis*.

RESULTS AND DISCUSSION

Cofactor requirement of the reductase

An experiment to determine the cofactor requirement showed that the reductase from the bottom fraction suspension had a specific requirement for NADPH. A specific activity of 4.71 ± 0.83 pkat/mg protein was obtained in the presence of 2 mM NADPH as against a value of 0.29 ± 0.19 pkat/mg protein in the presence of 2 mM NADH. These results are consistent with the earlier report [2] that $NADP^+$ (or NADPH) was a more effective cofactor than NAD^+ (or NADH) for the conversion of labelled HMG-CoA into rubber. The effectiveness of $NADP^+$ was apparently due to the presence of an endogenous $NADP^+$ reducing system in their enzyme preparation. The preference for NADPH as the cofactor appears to be a common feature of HMG-CoA reductase from a wide variety of sources, e.g. yeast [3], rat liver and intestinal mucosal cells [4] and *Pisum sativum* [5].

Localization of the reductase in latex

Latex is a cytoplasmic suspension of rubber particles and subcellular organelles [6]. Of the latter, the most numerous are the lutoids, followed by a much smaller number of Frey-Wyssling complexes [6]. The lutoids are vesicles, each surrounded by a single membrane, whereas the Frey-Wyssling complexes are double-membrane organelles containing carotene and lipid globules. Although the endoplasmic reticulum has been observed in electron micrographs of sections of the latex vessels [6], it is rarely observed in the bottom fraction of ultracentrifuged latex [7].

Measurement of reductase activity in the various fractions of ultracentrifuged latex obtained as described in the Experimental gave the following results (in pkat/mg protein): Frey-Wyssling zone, 1.03 ± 0.31; C-serum, 0.16 ± 0.01; bottom fraction, 4.40 ± 0.28. In terms of the reductase activity per ml of latex, the Frey-Wyssling zone and the C-serum each contributed 0.8 and 4.4% respectively of the total, while the

remainder was located in the bottom fraction. Reductase activity in the semi-solid rubber fraction was not determined. According to Hepper and Audley [2], however, little reductase activity was found in the rubber fraction. The data in the present study are in agreement with the results obtained using latex from seedling plants [2].

In order to ascertain whether the reductase was membrane-bound or was present in the liquid matrix of the organelles present in the bottom fraction, the pellet was resuspended in buffer A containing Triton X-100 (0.1%, w/v), a treatment known to lyse the membranes, in particular those of the lutoid particles [8]. The resulting suspension was centrifuged at 40000 *g* for 45 min and enzyme activity determined in the clear supernatant and in the sediment. The results presented in Table 1 show that the reductase activity was highest in the sediment. The activity in the supernatant was most likely to be due to the solubilized enzyme, since the specific activity and the percentage recovery increased (1.00 ± 0.08 pkat/mg protein and 33% respectively) when a higher level of Triton X-100 was used (1%, w/v). When sonication, instead of detergent, was employed to disrupt the organelles, very little enzyme activity was detected in the supernatant, whereas the pellet activity remained high (Table 2). In contrast, Hepper and Audley [2] observed that washing their bottom fraction (600 *g* pellet) in iso-osmotic buffer resulted in the loss of reductase activity in the washed pellet. They made no mention however, as to whether any reductase activity was recovered in the washings obtained.

From a kinetic study of the membrane-bound reductase obtained by sonication of the bottom fraction suspension as described in the legend to Table 2 (except that the centrifugation step was at 103 000 *g*), an apparent K_m of 56 μM for *RS*-HMG-CoA was obtained. This value falls within the range of K_m values reported for the rat liver enzyme [9] and is lower than the figure of 100 μM (*RS*-HMG-CoA) reported for the enzyme from *Pisum sativum* [5].

The localization of the reductase on any, or all, of the organelles mentioned above has not been established, mainly because of the practical difficulties encountered in obtaining the membranes of each type of organelle in the pure form. It is nevertheless possible to prepare a fraction enriched in Frey-Wyssling particles by centrifuging diluted latex [10]. Under these conditions, a small yellowish-orange pellet was obtained at the bottom of the centrifuge tube, while the lysed lutoids, which then became lighter and moved centripetally, separated as a whitish layer adjacent to the rubber layer. Enzyme activities in the enriched Frey-Wyssling fraction and in the lutoid fraction were 1.99 ± 0.39 pkat/mg protein and 10.78 ± 0.46 pkat/mg protein respectively. In terms of activity per ml of fresh latex, the Frey-Wyssling fraction contained *ca* 5% of the total activity, while the remainder was in the lutoid fraction. Taken together, these results indicate that the reductase is membrane-bound and that it is most likely to be bound to the lutoid particles in view of the preponderance of this organelle type in the bottom fraction. The occurrence of the reductase on the lutoid membranes would be consistent with the vacuolar nature of these organelles and their possible

Table 1. Effect of treatment of bottom fraction with Triton X-100 on HMG-CoA reductase activity

Enzyme fraction	Specific activity (pkat/mg protein)	Total activity (pkat)	%
1. Bottom fraction in buffer A	3.72 ± 0.24	61.4	—
2. Bottom fraction in buffer A + Triton X-100	3.58 ± 0.20	60.3	100
3. Supernatant (40 000 *g*)	0.70 ± 0.06	2.1	4
4. Pellet (40 000 *g*)	4.10 ± 0.06	30.3	50

Freshly prepared bottom fraction was resuspended in either buffer A or buffer A containing 0.1% (w/v) of Triton X-100. The latter suspension was mixed on a Vortex mixer for 1 min and allowed to stand in ice for 15 min. A portion of this suspension was then centrifuged at 40 000 *g* for 45 min to obtain the supernatant fraction and a pellet which was resuspended in buffer A. These fractions were assayed for reductase activity as described in the Experimental. Each value represents the mean ±s.d. of triplicate measurements.

Table 2. Effect of sonication of the bottom fraction on HMG-CoA reductase activity

Enzyme fraction	Specific activity (pkat/mg protein)	Total activity (pkat)	%
1. Bottom fraction suspension in buffer A	4.23 ± 0.69	103.0	—
2. Bottom fraction in buffer A (sonicated suspension)	1.75 ± 0.19	42.5	100
3. Supernatant fraction (40000 *g*)	0.13 ± 0.02	1.2	3
4. Pellet fraction (40 000 *g*)	2.87 ± 0.12	9.5	23

Freshly prepared bottom fraction was resuspended in buffer A. A portion of this was sonicated for 30 sec (Sonic 300 Dismembrator, Artek, U.S.A.). An aliquot was retained for enzyme assay while the remaining was centrifuged at 40 000 *g* for 45 min. The clear supernatant was removed and the pellet resuspended in buffer A. All the enzyme fractions were then assayed for reductase activity as described in the Experimental. Each value represents the mean ± s.d. of triplicate measurements.

ontogenic relationship with the endoplasmic reticulum [11]. In comparison, the enzyme from rat liver [12], and from *Pisum sativum* [13], is located mainly on the endoplasmic reticulum. The presence of reductase activity in the Frey-Wyssling fraction is also not unexpected, since these organelles contain carotene which they probably synthesize [6].

Stability of the reductase

As there have been reports that HMG-CoA reductase from rat liver is cold labile [14], the effect of collecting and fractionating latex at ambient temperature (23–27°) on enzyme activity was investigated. It was found that the activity was only 6% of that of the enzyme prepared under refrigeration (0–4°). Since latex collected and maintained at ambient temperature contains a much larger microbial population in a matter of a few hours [15], these results also indicate that the reductase activity observed in the bottom fraction is unlikely to be of microbial origin.

The stability of HMG-CoA reductase from various sources, e.g. rat liver [4, 16], yeast [17] and *Pisum sativum* [5], is dependent upon the presence of reduced thiol compounds. In the present study, the bottom fraction of ultracentrifuged latex was prepared without adding thiol, but in spite of this, reductase activity was stable over at least 5 hr, provided the preparation was kept in ice. The effect of storing the bottom fraction pellet at *ca* −15° was also examined. Such storage resulted in a 30% loss of enzyme activity within 24 hr and up to 50% loss of activity after 1 week. The residual activity, however, still remained at a workable level. Nevertheless, the effect of dithiothreitol on reductase activity was investigated and the result presented in Fig. 1 shows that the enzyme from freshly prepared bottom fraction was activated by this thiol and that the optimal concentration was 2 mM. Dithiothreitol (2 mM) also increased reductase activity in bottom fraction frozen for a week but not to the level of that in freshly prepared bottom fraction.

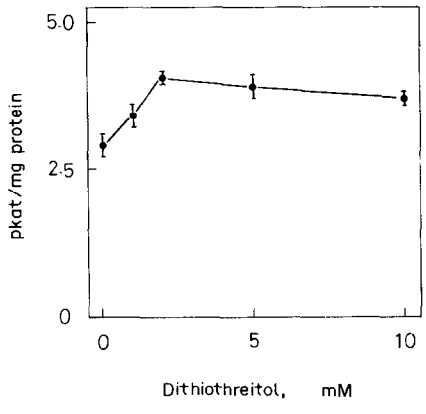

Fig. 1. Effect of dithiothreitol on HMG-CoA reductase activity. Freshly prepared bottom fraction was resuspended in buffer A from which dithiothreitol was omitted and enzyme activity was then assayed as described in the Experimental in the presence of the stated concentration of dithiothreitol. Each point on the graph represents the mean ±s.d. of triplicate measurements.

These results indicate that the reductase requires the presence of a free sulphydryl group for activity. Removal of such a group, e.g. by treatment with *p*-chloromercuribenzoate (at 1 mM), completely inhibits enzyme activity (Table 3). The fact that this thiol requirement is apparently not obligatory, can be explained by the presence of endogenous reduced thiol compounds in the latex [18]. The bottom fraction of ultracentrifuged latex in particular has been shown to contain substantial amounts of the thiol ergothioneine [19]. Thus these endogenous thiols can account for the better stability of the reductase enzyme preparation used in the present study. In comparison, Hepper and Audley [2] reported that the reductase in their preparations was labile even at −10° unless reduced glutathione, or cysteine, were present. It should be noted, however, that their latex was obtained from a seedling tree and that it was also diluted in buffer. Furthermore, ergothioneine was not detected in the latex obtained from their planting material [19].

pH optimum of the reductase

The enzyme was found to have maximal activity when assayed at pH 6.6–6.9 in 0.1 M phosphate buffer containing 2 mM dithiothreitol. This pH optimum is similar to that for the incorporation of HMG-CoA into rubber [2]. The pH optimum of the reductase from rat liver ranges from pH 6.5 to 7.5 [4, 14], while that of *Pisum sativum* is pH 6.8 in phosphate buffer [5].

Effect of mevalonate, CoA and hydroxymethylglutarate (HMG) on reductase activity

Mevalonate and CoA are both products of the reductase reaction and as shown in Table 3, these compounds did not affect the enzyme at the concentration of 2 mM tested. HMG, on the other hand, is a product of the cleavage of HMG-CoA by HMG-CoA hydrolase (EC 3.1.2.5). This enzyme has not been detected in either the C-serum or the bottom fraction of ultracentrifuged latex [Sipat, A. B., unpublished data]. Nevertheless, when added to the reaction mixture, HMG (2 mM) slightly decreased reductase activity (by *ca* 14%) in one experiment using a fresh enzyme preparation, but not in another in which stored bottom fraction was used as the enzyme source (Table 3). These results are of particular significance, since the substrate used in the enzyme assays was not purified and contained residual CoA and HMG (see Experimental). It therefore appears that the presence of small amounts of these compounds in the assay mixture did not significantly affect the measurement of reductase activity.

The effects of mevalonate, HMG and CoA on the incorporation of HMG-CoA into rubber have been studied previously [2]. It was found that mevalonate diminished the incorporation of HMG-CoA into rubber but this occurred only at high concentrations (above 3.2 mM). CoA also inhibited to a small extent the incorporation of both HMG-CoA and mevalonate into rubber but this effect apparently was not located at the reductase step. HMG, although not incorporated into rubber, was inhibitory. From the present study, it is probable that this inhibition by HMG is localized at the reductase step. In comparison the

Table 3. The effect of various compounds on HMG-CoA reductase activity

Compound added	Specific activity (pkat/mg protein) Expt 1	Expt 2
None	2.57 ± 0.14	1.21 ± 0.15
Mevalonate (2 mM)	2.64 ± 0.18	1.43 ± 0.29
HMG (2 mM)	2.20 ± 0.05	1.60 ± 0.34
CoA (2 mM)	2.85 ± 0.37	1.28 ± 0.06
Mg-ATP (4 mM each)	0.26 ± 0.06	0.39 ± 0.10
p-Chloromercuribenzoate (1 mM)	0.0	Not determined

The following results were obtained from two separate experiments. The bottom fraction was resuspended in buffer A from which dithiothreitol was omitted. Enzyme activity was then measured as described in the Experimental, except that each of the stated compounds was included in the reaction mixture. In experiment 2, a 4-day-old frozen bottom fraction was used as the enzyme source. Each value is the mean ± s.d. of triplicate measurements.

reductase from *Pisum sativum* was inhibited by CoA (at 0.025–0.5 mM) and HMG (1–10 mM), but not by mevalonate at 1 mM [5]. The enzyme from the liver and the intestinal mucosa of rat was reported to be sensitive to CoA [4, 15], but not to HMG [4]. The yeast enzyme has been shown to be sensitive to CoA and various CoA esters [17].

Inactivation of the reductase by magnesium–ATP

HMG-CoA reductase from rat liver microsomes was first reported to be inactivated by magnesium–ATP by Beg *et al.* [20] and their observation has since then been confirmed by several other workers [21]. The data in Table 3 show that the reductase from bottom fraction of ultracentrifuged latex is also sensitive to magnesium–ATP (4 mM). The inhibition appears to be independent of the presence of Mg^{2+} (Table 4). However, the bottom fraction of ultracentrifuged latex has been shown to contain endogenous Mg^{2+} at concentrations ranging from 370 to 520 μM [22] and this may be sufficient for ATP to take effect.

The inhibitory effect of magnesium–ATP on reductase activity in the bottom fraction can be partially or completely removed by washing the pellet. When a bottom fraction suspension (in buffer A) was sonicated as described in the legend to Table 2 and then followed by centrifugation at 103 000 *g* for 1 hr, the activity of the particulate reductase in the absence and presence of magnesium–ATP (4 mM each) was 10.58 ± 0.72 and 8.87 ± 0.33 pkat/mg protein respectively, i.e. an inhibition of *ca* 16% by magnesium–ATP. When the bottom fraction was washed in buffer A containing Triton X-100 (0.1%, w/v) as described in the legend to Table 1 and then followed by centrifugation at 103 000 *g* for 1 hr, the particulate reductase had an activity of 3.78 ± 0.31 and 3.87 ± 0.11 pkat/mg protein respectively in the absence and presence of magnesium–ATP (4 mM each), i.e. the inhibitory effect of the latter compound was completely removed. Thus a loosely bound factor in the bottom fraction appears to be required for the inhibitory effect of magnesium–ATP.

Table 4. Inactivation of HMG-CoA reductase activity by Mg-ATP

Addition	Specific activity (pkat/mg protein)	%
None	8.09 ± 0.51	100
ATP (4 mM)	0.82 ± 0.11	10
$MgCl_2$ (4 mM)	8.70 ± 0.22	108
ATP (4 mM) and $MgCl_2$ (4 mM)	0.62 ± 0.06	8

Freshly prepared bottom fraction was resuspended in buffer A and enzyme activity assayed as described in the Experimental in the presence of either $MgCl_2$ only, or ATP only, or both. Each value represents the mean ± s.d. of triplicate measurements.

It is possible that in these experiments, the mevalonate formed by the enzymic reaction was metabolized further in the presence of ATP. This, however, is unlikely, since firstly, mevalonate when added to the reductase reaction mixture could still be completely recovered (Table 5), and secondly, the enzymes which metabolize mevalonate (mevalonate kinase, EC 2.7.1.36 and phosphomevalonate kinase, EC 2.7.4.2) are predominantly in the C-serum fraction of ultracentrifuged latex [23]. The inhibitory effect of magnesium–ATP was also unlikely to be due to the activation of other HMG-CoA utilizing enzymes such as the lyase (EC 4.1.3.4) and the hydrolase (EC 3.1.2.5). The lyase has been shown to be located in the C-serum, but not in the bottom fraction [2], while hydrolase activity was not detectable in either fraction (see above). The inhibitory effect of ATP on reductase activity is of particular interest in view of

Table 5. Recovery of [^{14}C]mevalonate

Assay conditions	Radioactivity recovered as [^{14}C]mevalonate (dpm/hr/incubation mixture)	%
Complete	13 800 ± 390	100
Complete + ATP (4 mM)	13 800 ± 570	100
Boiled enzyme	12 800 ± 690	93

The incubation conditions and the measurement of radioactivity were as described in the Experimental for the assay of HMG-CoA reductase except that [^{14}C]mevalonate (50 nmol, 0.14 μCi/μmol) replaced the enzyme substrate.

the finding of Weeks and Kekwick [24], that this compound inhibited the incorporation of acetate into rubber when used at concentrations higher than 0.2 mM. These workers suggested that the ATP-sensitive step is located in the formation of mevalonate from acetyl-CoA. From the results of the present study, it is likely that this ATP-sensitive step is the reaction catalysed by HMG-CoA reductase.

The inactivation of the reductase by magnesium-ATP is a well-established phenomenon for the enzyme from rat liver and current evidence strongly suggests that its activity is regulated by phosphorylation-dephosphorylation [21]. The question, however, remains as to whether such a mechanism also operates in plants. The latex enzyme thus provides an opportunity to answer this.

EXPERIMENTAL

Collection of latex. Fresh latex was collected from nine regularly tapped (half-spiral cut, once every 2 days) trees of *H. brasiliensis* clone RRIM 600 (*ca* 15 years old) grown in the University Farm. The latex from each tree was allowed to run to waste for 3 min after tapping and then collected for the next 30 min into a flask surrounded by crushed ice. The contents of all flasks were pooled to yield 300–400 ml latex.

Fractionation of latex. The latex was fractionated by centrifugation at 40 000 *g* (R_{av} 5.74 cm) for 40 min, exclusive of acceleration and deceleration times. Four main fractions were obtained; the uppermost layer of semi-solid rubber, a small zone immediately below this containing some intact as well as fragments of Frey-Wyssling complexes (the Frey-Wyssling zone), the C-serum which corresponds to the cytosol and lastly the pellet at the bottom of the centrifuge tube, commonly referred to as the 'bottom fraction'. This mainly consists of lutoids, but some Frey-Wyssling complexes and other particles are present [7]. The upper zone containing the Frey-Wyssling complexes was carefully removed with a syringe and pooled with that from several other tubes. The resulting suspension was then centrifuged at 3000 *g* for 3 min to remove many of the rubber particles. The yellowish-orange sediment was resuspended in 0.1 M triethanolamine-HCl buffer (pH 7.1) containing 2 mM dithiothreitol (referred to as buffer A), and assayed for reductase activity as described below. The clear straw-coloured C-serum was removed by puncturing the tube and draining carefully into a beaker so as to exclude much of the rubber. The part of the tube containing the bottom fraction pellet was cut off completely and rinsed with ice-cold H_2O to remove the residual rubber. The pellet was then resuspended using a Potter-Elvehjem homogenizer in buffer A in the ratio of 1 ml of buffer to one bottom fraction pellet. This suspension was used as the enzyme source unless stated otherwise.

HMG-CoA reductase assay. The assay method was essentially as described in ref. [25]. Each reaction mixture contained: enzyme, 0.7–0.9 mg protein; NADPH, 0.5 μmol; *RS*-[3-^{14}C]HMG-CoA, 120 nmol (sp. act. 444 dpm/nmol) and buffer A in a final vol. of 0.2 ml. In some expts the dithiothreitol was omitted. Boiled enzyme preparation was used as the control. Incubations were carried out at 30° for 30 min and terminated with 25 μl 10 M HCl. The mevalonolactone, in a 100 μl aliquot of the protein-free supernatant, was isolated by TLC [25]. The zone of R_f 0.46–0.73 on the TLC was scraped into a scintillation vial and 1 ml H_2O added to resuspend the Si gel. Radioactivity was measured by liquid scintillation counting (counting efficiency 92–94%) in toluene-Triton X-100 (2:1, v/v) solvent containing 0.5% 2, 5-diphenyloxazole. The recovery of mevalonolactone from the TLC was 95–98%. Enzyme assays were done in triplicate and the measurements expressed are the mean ± s.d. in units of pkat (pmol mevalonate formed/sec). The enzyme activity was linear within 30 min incubation time and up to 1.25 mg enzyme protein under the above-mentioned conditions. The enzyme was satd at *ca* 300 μM *RS*-HMG-CoA.

[^{14}C]HMG-CoA was synthesized as described in ref. [26] and was used without further purification. It was estimated that on using this preparation, each reaction mixture (see above) also contained 24 nmol [^{14}C]HMG and 6 nmol CoA. Their presence did not significantly affect the measurement of enzyme activity (see Results and Discussion).

Protein measurement. Protein was determined by the method of ref. [27] after pptation with TCA at a final concn of 10% (v/v). BSA was used as the standard.

Chemicals. CoA (the lithium salt), HMG and bovine serum albumin were obtained from Sigma. [3-^{14}C]HMG and [2-^{14}C]mevalonate (DBED salt) were purchased from New England Nuclear.

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