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PARTIAL PURIFICATION AND CHARACTERIZATION OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE FROM THE LEAVES OF GUAYULE (*PARTHENIUM ARGENTATUM*)

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Key Word Index—*Parthenium argentatum*; Asteraceae; guayule; leaves; HMG-CoA reductase; purification; properties.

Abstract—3-Hydroxy-3-methylglutaryl coenzyme A reductase has been isolated and was partially purified from the leaves of *Parthenium argentatum*. The enzyme was found to be associated both with the cytosol and the chloroplasts. Ten mM dithiothreitol was essential to prevent loss of activity. Optimum activities of cytosolic and chloroplastic fractions were observed at pH 7.0 and 7.5 respectively. Preincubation of the reaction mixtures with CoA, acetyl-CoA, σ-phenanthroline and iodoacetamide resulted in the progressive loss of enzyme activity. 3-Hydroxybutyrate and mevalonate also inhibited the enzyme. The Michaelis constants of the enzyme for HMG-CoA and NADPH were 0.25 and 0.31 mM respectively for the cytosolic enzyme, while those for the chloroplastic enzyme were 0.018 and 0.42 mM respectively. Inhibition studies indicated that hydroxybutyrate was a competitive inhibitor with respect to HMG-CoA. The inhibition of mevalonate was competitive with HMG-CoA and non-competitive with NADPH.

INTRODUCTION

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (mevalonate: NADP oxidoreductase acylating CoA, EC 1.1.1.34) catalyses the reduction of HMG-CoA to mevalonate and this reduction is an early reaction in the biosynthesis of isoprenoid compounds [1–3]. It has been suggested that the formation of mevalonate from HMG-CoA is an important rate-limiting step in tissues which biosynthesize rubber [4–7]. HMG-CoA reductase has been studied only in a limited number of plant tissues such as sweet potato roots, pea seedlings and radish seedlings [8–10].

In the last ten years there has been a great revival of interest in guayule (*Parthenium argentatum* Gray) as a source of natural rubber. Although the agronomic and breeding traits of this crop are fairly well known [11, 12], very little attention has been paid to the basic physiological and biochemical processes. The present paper reports on the intracellular distribution of HMG-CoA reductase in leaves of guayule and on some properties of the partially purified cytosolic enzyme.

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RESULTS AND DISCUSSION

HMG-CoA reductase was partially purified by the procedure summarized in Table 1. About 33% of the original activity was recovered in the fractions from DEAE-cellulose. The purified enzyme was unstable at both 0° and 30°, unless the resuspension medium was supplemented with 10 mM dithiothreitol (DTT) when there was no loss of activity over several hours at 0°. There was almost a two-fold increase in the enzyme activity in the presence of 10 mM DTT (Fig. 1). The results indicated that the reductase activity was either protected or stimulated by the presence of a thiol compound.

Because of the paucity of information on the subcellular localization of HMG-CoA reductase, experiments were carried out to determine the activities in the par-

Table 1. Partial purification of HMG-CoA reductase

Fraction	Total activity (nkat)	Specific activity (pkat mg prot^{-1})	Yield (%)
Leaf extract	163	67	100
$(NH_4)_2SO_4$, 30–60% saturation	118	95	72
Sephadex G-200	100	188	61
DEAE-cellulose	53	422	33

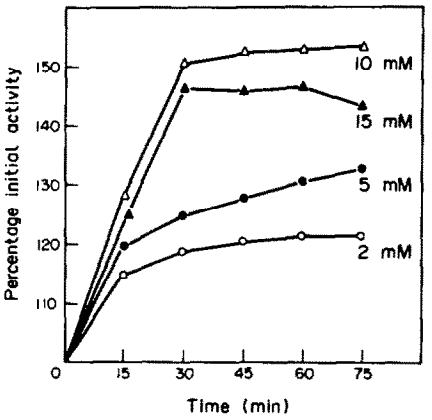

Fig. 1. Relative activity of HMG-CoA reductase from *P. argentatum* in presence of different concentrations of DTT.

ticulate and non-particulate fractions. The isolated chloroplasts purified through a Percoll gradient were 90% intact as revealed by ferricyanide reduction activity and the sucrose density gradient profile indicated that activity was to be found in them (Fig. 2). Trials with purified mitochondria indicated that these organelles had no detectable reductase activity. The findings strongly support the occurrence of compartmented pathways of isoprenoid biosynthesis in guayule cells. $^{14}CO_2$ and ^{14}C-MVA feeding experiments with maize seedlings showed that the enzymes of isopentenyl pyrophosphate (diphosphate) biosynthesis were located in more than one cellular compartment [13]. Our results are in agreement with the views of Arebalo and Mitchell [14] that there are two separate forms of HMG-CoA reductase in plant cells, one inside the chloroplast and one outside the chloroplast.

The activity of the cytosolic reductase is optimum at pH 7.0 while that of the chloroplast enzyme is 7.5. The Michaelis–Menten constants of the reductase with varying concentrations of HMG-CoA and NADPH revealed that the cytosolic enzyme had a higher K_m for HMG-CoA (0.25 mM) than that of the chloroplasts (0.018 mM) while K_m (NADPH) was not significantly different for the two

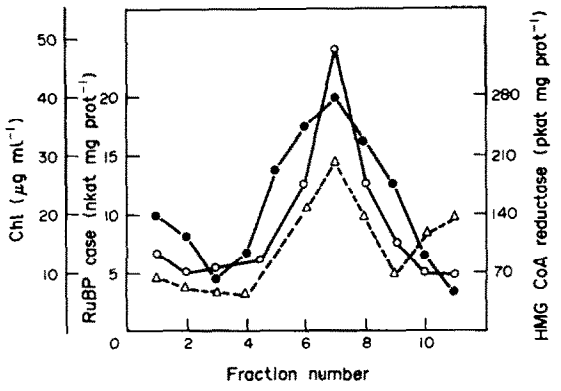

Fig. 2. HMG-CoA reductase (△ - - △) activity profile along with those of ribulose bisphosphate carboxylase activity (●——●) and chlorophyll (○——○) content obtained on sucrose density gradient centrifugation of the chloroplast preparation from *P. argentatum* leaves.

reductases (cytosolic form, 0.31 mM. chloroplastidic form, 0.42 mM). V_{max} values with respect to HMG-CoA were, however, high with the chloroplast reductase (chloroplastidic form, 333 pkat mg prot^{-1}; cytosolic form, 127 pkat mg prot^{-1}). The apparent K_m values of the cytosolic reductase for HMG-CoA are slightly high compared with those reported for other plants. The present data however provide substantial evidence that the kinetic properties of cytosolic and plastid reductases are quite different from each other.

Experiments with inhibitors were performed with the cytosolic enzyme. Incubation of the enzyme with σ-phenanthroline and iodoacetamide, which are known inhibitors of mevalonate metabolism, resulted in progressive loss of enzyme activity (Fig. 3 and Table 2). These results support the possibility that these inhibitors may destabilize the enzyme probably through metal chelation or by reacting with sulphydryl groups. The progressive loss of inactivation of the enzyme by preincubating with CoA and acetyl-CoA is of greater significance (Fig. 3). Further studies are in progress to understand the mode of inhibition. Significant loss of the reductase activity was noticed when hydroxybutyrate (HBA) and mevalonate were added to the reaction mixtures (Fig. 4). The inhibition of bacterial HMG-CoA reductase by hydroxybutyrate has been previously reported [15]. Our results indicate that this inhibitor competes with HMG-CoA thus rendering the site to HMG-CoA inaccessible. For

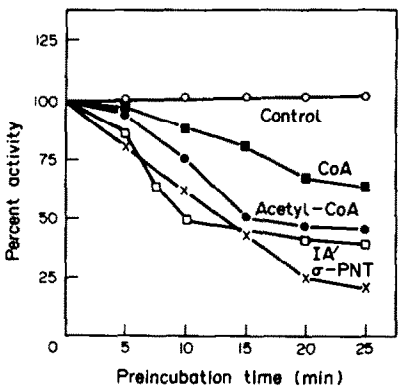

Fig. 3. Course of inactivation of HMG-CoA reductase by preincubating the cytosolic enzyme with certain inhibitors and cofactors (IA: iodoacetamide; σ-PNT: σ-phenanthroline).

Table 2. Effect of certain inhibitors and cofactors on HMG-CoA reductase activity from the cytoplasm

Experiment	Enzyme activity (% control)
Control	100*
+ Coenzyme A (0.5 mM)	60
+ Acetyl CoA (0.5 mM)	55
+ Iodoacetamide (0.4 mM)	47
+ σ-Phenanthrolene (0.5 mM)	40
+ p-Phydroxymercuribenzoate (0.1 mM)	53

Inhibitors and the cofactors were added to the enzyme assay mixture described in the text.

*100% = 477 pkat mg protein^{-1}.

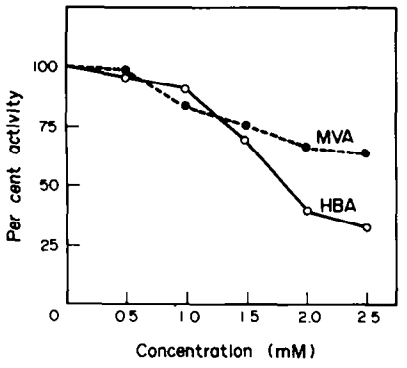

Fig. 4. Mevalonate and hydroxybutyrate inhibition of cytosolic HMG-CoA reductase activity.

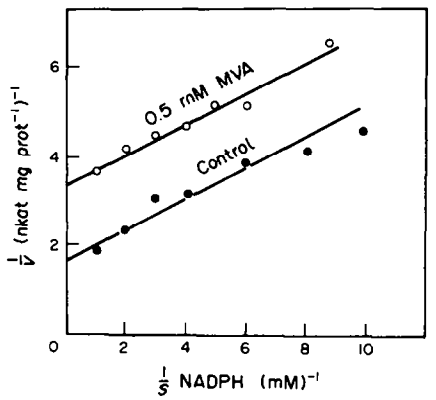

Fig. 5. Mevalonate inhibition of cytosolic HMG-CoA reductase at different concentrations of NADPH.

instance, with 0.5 mM HBA, the reaction was inhibited by about 65% with an increase in K_m (HMG-CoA) from 0.25 to 0.46 mM. Certain 3-hydroxy-3-methylcarboxylic acids that are structurally similar to HMG-CoA are competitive inhibitors of the reductase [15]. Product inhibition studies on the reductase indicated that mevalonate gives competitive inhibition with respect to HMG-CoA. K_m (HMG-CoA) was increased (0.67 mM) in the presence of 1 mM mevalonate when compared to the control experiment (0.25 mM). The inhibition with NADPH was non-competitive (Fig. 5). The non-competitive inhibition of mevalonate in this type of reaction sequence would indicate that the product binds to a form of HMG-CoA reductase which differs from that form which binds the substrate. This feed back inhibition of the reductase may play a significant role in the regulation of the enzyme.

EXPERIMENTAL

Materials. (*RS*)-Hydroxy-3-[3-^{14}C]methylglutaryl coenzyme A (58 mCi/mmol) was obtained from Amersham International, U.K. Percoll was purchased from Pharmacia (Uppsala, Sweden) and DEAE-cellulose (DE-52) from Whatman Biochemicals Ltd., (Maidstone, U.K.). HMG-CoA, mevalonate and all other biochemicals were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A.

Guayule (*Parthenium argentatum* Gray) plants were grown in the field under local conditions. The day/night temp. regime was 35°/22° and the average photon flux density was 1800 μE m^{-2} sec^{-1}.

Preparation and partial purification of the enzyme. Fully expanded leaves were used. The leaf tissue (50 g) was homogenized in 50 ml of extraction medium containing 50 mM KPi buffer, pH 7.5, 30 mM mercaptoethanol, 5 mM DTT, 5 mM $MgCl_2$, 1 mM EDTA and 1% polyvinylpyrrolidone-40 (PVP-40). The slurry was squeezed through two layers of muslin cloth and the filtrate centrifuged at 200 *g* for 2 min to remove debris. The supernatant was subjected to two centrifugations first at 1000 *g* for 3 min to remove the chloroplasts followed by a 45000 *g* for 20 min. The protein precipitating between 30 and 40% satn was obtained by adding solid $(NH_4)_2SO_4$ to the 45000 *g* supernatant. The sedimented protein was recovered by centrifuging at 5000 *g* for 20 min. The protein was dissolved in the blending medium and applied to a column (20 × 2 cm) of Sephadex G-200 which has been equilibrated with 15 mM KPi buffer, pH 7.5 and 10 mM DTT. Protein was eluted at a flow rate of 0.5 ml min^{-1} and 5 ml fractions were collected. Most of the HMG-CoA reductase activity eluted in fractions 6–9 excluded protein. The fractions were combined and applied to a column (20 × 2 cm) of DEAE-cellulose previously equilibrated with 15 mM KPi buffer, pH 7.5 and 10 mM DTT. The enzyme was eluted with the same buffer. Fractions of 5 ml were collected and assayed for HMG-CoA reductase activity. For storage, active fractions were resuspended in 50 mM KPi buffer, pH 7.5 containing 10 mM DTT and stored at 0° prior to use. All the operations were carried out at 0–4°.

Isolation of chloroplasts. The leaves were harvested and illuminated for 15 min prior to grinding. All the operations were carried out at 0–4° in a cold room. The leaves were cut into strips and homogenized in a semifrozen grinding medium which consisted of 0.53 M sorbitol, 10 mM PPi, 5 mM $MgCl_2$, 1% PVP, 0.5 mM DTT and 2 mM sodium arsenate. The slurry was squeezed through two layers of cheesecloth and the filtrate was centrifuged at 250 *g* for 5 min to remove cell debris; the supernatant was further centrifuged at 2500 *g* for 10 min. Chloroplasts from the crude pellet were then purified in a Percoll gradient, as described by Mourioux and Douce [16]. The purified chloroplasts were suspended in a medium containing 0.33 M sorbitol, 50 mM HEPES (pH 7.6), 1 mM $MgCl_2$ and 1 mM $MnCl_2$. The suspension was then layered on to a sucrose gradient comprising 1.5, 1.0 and 0.75 M sucrose in 10 mM Tricine–KOH (pH 7.6), centrifuged at 2000 *g* for 5 min and then at 10000 *g* for 10 min. Fractions of 1.5 ml were collected for enzyme assays.

Enzyme assay. The assay system contained (total vol. 1.0 ml) 50 mM KPi buffer (pH 7.2), 3 mM NADP, 20 mM glucose-6-phosphate, 10 mM DTT, 0.25 mM (*RS*)-[3-^{14}C]HMG-CoA (1.8×10^5 dpm/μmole), 2 units/ml glucose-6-phosphate dehydrogenase and the enzyme. The reaction was started by adding HMG-CoA. The reaction tubes were incubated with shaking for 30 min at 30°. The reaction was stopped by adding 20 μl of 4 M HCl and 20 μl of 5% (*RS*)-mevalonolactone and the mixture allowed to stand at room temp. for 15 min. After centrifugation, the supernatant was analysed for MVA-lactone as described by Russel [3]. Protein was determined by a modified Lowry [17]. Chlorophyll was measured by the procedure of Arnon [18]. The activities of ribulose bisphosphate carboxylase were assayed according to Lilley and Walker [19].

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