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3-Hydroxyl-3-methylglutaryl coenzyme A synthase activity in *Hevea braziliensis* and its possible role in regulation of rubber synthesis

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ABSTRACT: The activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase from rubber (*Hevea brazilliensis*) latex and from fractions obtained by centrifugation was determined using spectrophotometric method. The enzyme was present in all the fractions except rubber but was most abundant in C-serum. The enzyme obeys Michaelis-Menten kinetics and has a Km value of 10 mM for acetyl CoA. The enzyme is inhibited by several divalent cations, p-chloromercuribenzoate and dithiothreitol but activated by EDTA. Cd2+ is the most potent inhibitor among all the cations examined. Diurnal variation in enzyme activity was observed. The positive correlation between enzyme activity and rubber content of the latex suggests that the enzyme regulates the biosynthesis of rubber in the latex.

Key Words: HMG-CoA; HMG-Co A synthase; Rubber (*Hevea brazilliensis*); Rubber synthesis; Enzyme kinetics.

Introduction

The enzyme HMG-CoA synthase (3-hydroxy-3-methyl-glutaryl-CoA, acetoacetyl-CoA lyase (CoA acetylating), EC 4.1.3.5) has been extensively investigated in animals (1 – 6). It catalyses the reaction:

Acetyl-CoA + acetoacetyl-CoA $\rightarrow$ 3-hydroxy-3-methyl glutaryl-CoA + CoA

which occurs in isoprenoid and cholesterol synthesis. The enzyme in birds and animals has been purified and characterized (1 – 5) and is found to be located in both the cytosol and mitochondria (3,5). the enzyme from the two compartments of the avian tissue differ in properties and in their response to magnesium (5 – 7). Cytosolic HMG-CoA synthase exists in isoenzymic forms with distinct characteristics, while the mitochondrial form is involved in ketogenesis. Compounds which inhibit the enzyme have been reported (8) and the active site of the enzyme has been identified (5,9).

In mammals, cholesterol synthesis has been shown to be regulated by HMG-CoA synthase in association with HMG-CoA reductase (9 – 11) and the two enzymes function in concert in response to the supply of substrate for cholesterol synthesis in rat liver and adrenal (10). The regulatory activity of HMG-CoA synthase has been reported in rat adrenal gland and in rat brain (12). In yeast the activity of the enzyme is decreased when there is excessive ergosterol. (13).

The presence of HMG-CoA synthase and HMG-CoA reductase in *Hevea brazilliensis* latex has been reported 914). The enzyme in the latex catalyses the conversion of acetyl-CoA to isoprene in a manner similar to the synthesis of cholesterol in mammals. Attempt have been made to purify and study the nature of this enzyme in plants (20) but so far very little is known (15). While the nature of HMG-CoA reductase in *Hevea brazilliensis* and its gene expression have been studied in some details (16), HMG-CoA synthase and its possible role in regulation of rubber synthesis has not been studied.

The present study reports the activity of HMG-CoA synthase in various fractions obtained by centrifugation of latex and extracts of leaves. The factors affecting the activity of the enzyme from the cytosolic fraction (C-serum) of latex are also elucidated.

Materials and Methods

Plant Materials

Fresh latex was collected from the half-spiral cut, once every 2 days from *Hevea brazilliensis* (local cultivar) grown in plantation. The 20 year old trees were tapped at 1.00 a.m. and the latex was left in tapping cup on ice and collected at 6 a.m. The latex was fractionated by centrifugation in pre-cooled rotor at 30,000g for 30 min. Four fractions were obtained. The top rubber layer; the zone next to it containing Frey-Wyssling particles; the clear C-serum (cytosolic fraction), and the bottom fraction (pellet) containing lutoids and other particles. The C-serum was removed with syringe and the bottom fraction obtained by cutting the tube. All fractions were assayed for HMG-CoA synthase activity, except rubber. For assay of diurnal variation in the HMG-CoA synthase activity, samples of latex were collected from the same set of rubber trees at various times in a 24h period ranging from 6 a.m. to 6 a.m. the next day at 4 hours interval. The extracts from leaves of the seedlings were prepared as described by Suvachittanont and Wititsuwannakul (15). All chemicals and reagents used were products of Sigma Chemical Company and were of analytical grade.

HMG-CoA synthase assay

The enzyme activity was determined as described by Clinkenbeard et al. (1) using the decrease in absorbance of acetoacetyl-CoA at 300 nm (5). HMG-CoA lyase was assayed as described by Suvachittanont and Wititsuwannakul (15).

Protein assay

Protein contents were estimated using dye-binding method (17).

Rubber content of latex

Two hundred millilitres of latex was dried at 75^{o}C for two days to a constant weight and the rubber was weighed.

Results and Discussion

A spectrophotometric method which involves following the utilization of acetoacetyl-CoA at 300 nm was used in this study to assay HMG-CoA synthase from the latex of *Hevea brazilliensis*. This method has been reported to be inappropriate for assaying synthase activity in *Hevea brazilliensis* because of high absorbance of serum at this wavelength (15). Such problem was not encountered in this study. The HMG-CoA synthase activity was 35 nkat in the latex. The activity is several times higher than that found for HMG-CoA reductase in the latex (16).

HMG-CoA lyase hydrolyses HMG-CoA to acetyl-CoA and acetoacetate and so can interfere in the assay of synthase (12). In this work, no lyase activity was detected in different fractions separated. the present finding of the absence of lyase is in agreement with the work of Hopper and Audley (18) who observed very little activity of lyase in the latex from seedlings. The absence of lyase in C-serum may be compared to similar finding for cytosolic fraction from rat brain (12). In chicken liver, there was little HMG-CoA activity in the cytosolic fraction but more activity was found in mitochondria (3).

The relative volumes of various fractions obtained by centrifugation were, 50 for rubber and 13 for bottom fraction. The activity of synthase in each of these fractions was determined except that of the rubber due to practical difficulties associated with the rubber itself. the activity of the enzyme per cm^3 of latex was very close to activity per cm^3 of C-serum and this suggests that most of the activity is in the C-serum. Only little activity was found in Frey-Wyssling fraction (2 – 4% of the total) compared to other fractions while serum and bottom fractions had 70% and 26% of the total activity in the latex respectively (Table 1). The specific activity in the C-serum is 1220 nkat per mg protein and that of bottom fraction is 300 nkat per mg protein, indicating that the activity in the bottom fraction is about one fourth of the one in the C-serum. C-serum is cytosolic fluid and the bottom fraction contains membrane bound particles. These findings are similar to those found in chicken liver and rat brain where cytosolic and mitochondrial HMG-CoA synthases have been reported (1,3,5,12). In contrast to latex, the major proportion of synthase enzyme from other sources is found in mitochondria (1,12). It appears, therefore, that the enzyme of C-serum of the latex is responsible for isoprenoid synthesis, mainly rubber, like the cytosolic enzyme from other sources, while enzyme from the bottom fraction was involved in ketogenesis like that in mitochondria.

Table 1: Distribution of HMG-CoA synthase in different fractions of latex separated by centrifugation.

Fraction	Volume (%)	Enzyme activity (%)	Specific activity (nkat mg^{-1} prot.)
Rubber	50	–	–
C-serum	38	70	1220 ± 50
Frey-Wyssling	(1 – 2)	4	440 ± 30
Bottom	13	26	300 ± 20

The optimum pH found for enzyme is 8.2 and this is within the pH range of 8 to 8.2 found for synthases from other sources (1,5,12,15). Although the activity of the synthase under study was low when assayed at pH 6 and 9, and the pH of C-serum is between 6.5 and 7.0, the difference in pH suggests that the physiological reaction may occur in the micro-environment which provides the optimum pH for the enzyme. It has been reported that acetoacetyl-CoA inhibits the enzyme from chicken liver (4). In this study, there was no inhibitory effect of acetoacetyl-CoA on C-serum enzyme.

The enzyme obeys Michaelis-Menten kinetics when acetyl-CoA was the variable substrate and the concentration of acetoacetyl-CoA was constant at 0.06 mM. The apparent K_m for acetyl-CoA is 10 mM. This K_m vakue is very high when compared to that reported for chicken liver enzyme (0.1 mM) (1) and nearer to that reported by Suvachittanont and Wititsuwannakul (15) for C-serum enzyme. V_{max} found for the enzyme is 820 nkat per mg protein.

Cholesterol synthesis in mammals can be regulated through HMG-CoA reductase and HMG-CoA synthase. It is therefore important to study the factors that affect the activity of C-serum synthase. Table 2 shows the effect of various concentrations of some compounds on the synthase activity. The enzyme is inhibited by Cd^{2+}, Mg^{2+}, Pb^{2+}, Fe^{2+} and PCMB but activated by EDTA. The most potent inhibition of the enzyme is by lead ion (Pb^{2+}) and the least is by Fe^{2+}. The inhibition of Mg^{2+} is similar to that reported for rat brain HMG-CoA synthase (12) but differs from that of rat liver cytosol which is activated by Mg^{2+}. However, mitochondrial and cytosolic enzymes from other mammalian tissues have been shown to be sensitive to Mg^{2+} (1, 4). It has been reported that sulphydryl group in avian liver HMG-CoA synthase makes the enzyme sensitive to inhibition by low concentration of Cd^{2+} (0.05 mM) (19) and this is reversed by EDTA. The enzyme from C-serum studied was inhibited by low concentrations of Cd^{2+} (Table 2) and

the inhibition was reversed by EDTA. p-Chloromercuribenzoate (PCMB) also inhibited the enzyme. It seems, therefore, that the C-serum synthase contains sulphydryl groups which are essential for enzyme activity. However, dithiothreitol (DTT) which is known to stabilize sulphydryl containing enzymes inhibited C-serum synthase (Table 2).

Table 2: Effects of different compounds on the activity of HMG-CoA synthase from C-serum of latex.

Addition to assay	Concentration of effectors (mM)	% Activity remaining
None		100
EDTA	0.10	130
$MgCl_2$	5.0	97
$MgCl_2$	10.0	73
$CdCl_2$	0.005	48
$CdCl_2$	0.05	33
$CdCl_2$	0.10	11
$PdCl_2$	0.005	98
$PdCl_2$	0.05	68
$FeSO_4$	0.05	84
Compound + EDTA		
$CdCl_2$	0.05	93
PCMB	0.5	3
DTT	1.0	96
DTT	4.0	66
DTT	8.0	58

Activity of the enzyme fluctuated with time for tapping (Fig. 1), being highest at 22h and lowest at 6h and 14h. This type of diurnal rhythm has been reported for synthase from rat liver cytosol (2) and clone RRIM600 enzyme (15). This suggests that HMG-CoA synthase may be a controlling factor in rubber synthesis as suggested for reductase (14, 18).

HMG-CoA synthase activity has been reported in leaves of spinach, peas and beans (20). There was little activity of the enzyme in the leaves of rubber trees studied. When the extract of the leaves were fractionated into cytosol, mitochondria and chloroplasts, the activity of the enzyme was not detected in any of the fractions. Similar results have been reported for *Hevea brazilliensis* (15). Although the leaves contain laticifers and used to make rubber, the very high activity of synthase of C-serum is understandable. While the C-serum originates almost entirely from the cytoplasm of a particular cell type (laticifer) whose major function is to synthesize polyisoprene, the synthesis of isoprenoid is a very minor activity of the leaves (15).

In order to find out if the synthase plays any role in the regulation of rubber synthesis, the total activity and total rubber content of 25 samples of latex obtained by tapping the same group of 10 trees were determined. The two parameters correlated fairly well (Fig. 2). This result indicates that the synthase plays some role in regulation of rubber formation. However, the specific activity of synthase decreased as the rubber content increased and this is consistent with the finding that high level of cholesterol inhibits the activity of HMG-CoA synthase and reductase in human hepatoma HopG2 cells (11).

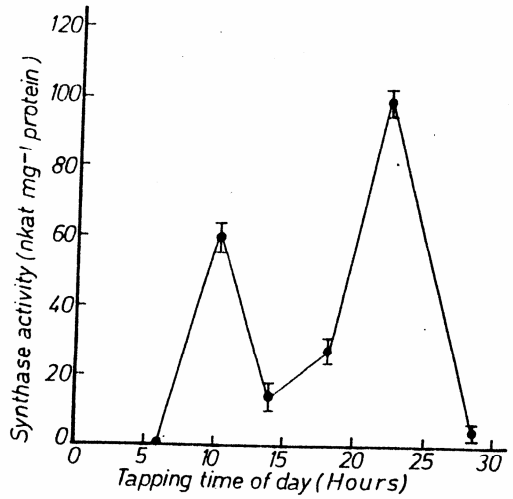

Fig. 1: Diurnal variation of HMG-CoA synthase in C-serum of latex. Each point represents the mean of 4 determinations ± S.E.M.

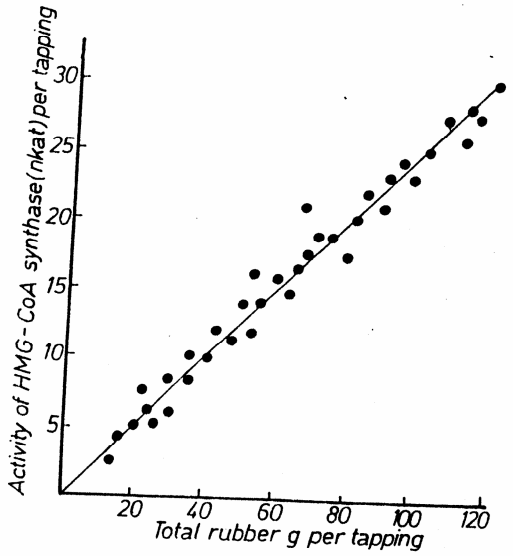

Fig. 2: Correlation between total activity of HMG-CoA synthase and dried rubber content in individual tapping.

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