Pergamon

0031-9422(95)00310-X

Phytochemistry, Vol. 40, No. 3, pp. 757–761, 1995

0031-9422/95 $9.50 + 0.00

3-HYDROXY-3-METHYLGLUTARYL-COENZYME, A SYNTHASE IN *HEVEA BRASILIENSIS*

WALLIE SUVACHITTANONT* and RAPEPUN WITITSUWANNAKUL

Biochemistry Department, Faculty of Science, Prince of Songkla University, Hat Yai, Songkla, Thailand 90112

(*Received in revised form* 6 *March* 1995)

Key Word Index—*Hevea brasiliensis*; Euphorbiacea; rubber; enzyme; hydroxymethylglutaryl-CoA synthase.

Abstract—3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase activity in rubber (*Hevea brasiliensis*) latex and in fractions obtained by centrifugation was determined using a radiochemical method. The enzyme was found in both C-serum and bottom fraction, but most was in the C-serum. *Hevea brasiliensis* leaves showed very low enzyme activity. HMG-CoA synthase in C-serum was found to be free of HMG-CoA lyase. The enzyme in C-serum was stable at − 70°. The formation of HMG-CoA did not depend on a supply of exogenous acetoacetyl-CoA, and the rate of formation was increased as the concentration of acetyl-CoA was increased. The K_m for acetyl-CoA was 9 mM. The enzyme in C-serum was inhibited by several divalent cations, *p*-chloromercuribenzoate and dithiothreitol. Diurnal variation in the activity of the enzyme in both C-serum and extracts of leaves was observed. A positive correlation between the enzyme activity and the rubber content of the latex (correlation coefficient of + 0.81) suggests that the enzyme regulates the synthesis of rubber in the latex.

INTRODUCTION

The enzyme HMG-CoA synthase [3-hydroxy-3-methylglutaryl-CoA acetoacetyl-CoA lyase (CoA-acetylating); EC 4.1.3.5] has been extensively investigated in animals [1–8]. It catalyses the reaction:

$$\text{acetyl-CoA} + \text{acetoacetyl-CoA} + H_2O \rightarrow \text{3-hydroxy-3-methylglutaryl-CoA} + \text{CoA}$$

which occurs in isoprenoid and cholesterol synthesis.

This enzyme in birds and mammals has been purified and characterized [1–6]; it has been shown to be localized in both cytosolic and mitochondrial fractions [1, 2, 4, 6]. The enzymes in the two fractions from avian tissue differ in their isoelectric points, molecular and catalytic properties, and in their responses to magnesium ion [6–9]. Cytosolic HMG-CoA synthase exists as an isoenzyme with distinct characteristics. Mitochondrial HMG-CoA synthase is involved in ketogenesis. Materials which inhibit the enzyme have been reported [10–12]. The active site of the enzyme has been identified [8, 12]. The gene encoding the enzyme has been located, cloned and sequenced; the expression of the gene has also been studied [7, 13, 14].

In mammals, cholesterol synthesis has been shown to be co-ordinatedly regulated by HMG-CoA synthase and HMG-CoA reductase activities [7, 15–18]. The two enzymes function in concert in response to the supply of substrate for cholesterol biosynthesis in rat liver and adrenal gland [3, 16]. Multiple regulatory elements in the promoter for hamster HMG-CoA synthase in sterol synthesis have been demonstrated [17]. The regulatory activity of HMG-CoA synthase in rat adrenal gland and in rat brain have also been reported [3, 19]. In yeast, HMG-CoA synthase activity has been shown to be decreased when ergosterol is in excess and the enzyme is also transcriptionally regulated [20].

It has been demonstrated that HMG-CoA synthase is also present in plants [21–23]. Lynen reported the presence of both HMG-CoA synthase and HMG-CoA reductase in *Hevea brasiliensis* latex [21]. In this latex, the enzyme HMG-CoA synthase also catalyses the utilization of acetyl-CoA for isoprene synthesis in a similar fashion to the synthesis of cholesterol in the mammal. Attempts have been made to purify, and to study the nature of, this enzyme in plants [22, 23], but so far, little is known. While the nature of HMG-CoA reductase in *H. brasiliensis*, and its gene expression, have been examined in some detail [24–27], HMG-CoA synthase, and its possible role in the regulation of rubber biosynthesis, has not been studied. In the present work, HMG-CoA synthase activity in various fractions obtained by centrifugation of the latex, and in extracts of the leaves, was determined. Factors affecting HMG-CoA synthase in the cytosol (C-serum) of latex were also investigated.

RESULTS AND DISCUSSION

Spectrophotometric and radiochemical methods are commonly employed to assay HMG-CoA synthase

*Author to whom correspondence should be addressed.

[1, 6]. The former, which involves following the utilization of acetoacetyl-CoA by measurements at 300 nm [6] could not be used with C-serum, because of the relatively high absorbance of the serum at this wavelength; the radiochemical method was therefore selected. The HMG-CoA synthase activity was 22 ± 3 nkat ml^{-1} in latex (three determinations). This activity is several times higher than that found for HMG-CoA reductase in latex [24].

HMG-CoA lyase hydrolyses HMG-CoA to acetoacetate and acetyl-CoA and so can interfere in the assay of HMG-CoA synthase. C-serum, bottom fractions, and the latex itself, were found to be free of the lyase because no [3-^{14}C]HMG-CoA was decomposed, as determined by the method of Shah [19] after incubation for 15 min. This time is much longer than that used for HMG-CoA synthase determination. The observation that these fractions did not show any HMG-CoA lyase activity is in good agreement with the work of Hepper and Audley [28], who demonstrated little HMG-CoA lyase activity in latex from seedlings, when a much longer incubation time (3 hr) was used. The absence of the lyase from C-serum may be compared with a similar finding for the cytosolic fraction from rat brain [19]. In chicken liver, there is little HMG-CoA lyase activity (4% of the total) in the cytosolic fraction, but substantial activity was in the mitochondria [4].

The relative volumes of the fractions obtained by centrifugation (see Experimental) were approximately: rubber, 51; Frey–Wyssling particles, 1–2; C-serum, 37; bottom fraction, 14. The activity of the enzyme in these fractions was determined, except that in the rubber, owing to a practical difficulty related to the rubber itself. The activity of the enzyme per ml of latex was quite close to the activity per ml of C-serum, suggesting that most of the activity is in the C-serum.

Only a little enzymic activity was found in the Frey–Wyssling fraction (5–10% of the total) compared to the other fractions. Contamination with C-serum might have contributed to some of the activity. The specific activity of the enzyme in the Frey–Wyssling fraction was 550 ± 50 pkat mg^{-1} protein (three determinations), C-serum and bottom fraction had *ca* 70 and 26% of the total activity in the latex, respectively (Table 1). Some of the activity in the bottom fraction would arise from contamination with serum. The specific activity of the enzyme in the bottom fraction was only one third that of the serum (1 130: 370 pkat mg^{-1} protein). The fact that the C-serum is a cytosolic fluid and that the bottom fraction contains mainly lutoids, which are membrane-bound particles, makes this finding similar to those in chicken liver and rat brain, where both cytosolic and mitochondrial HMG-CoA synthase have been demonstrated [1, 2, 4, 6, 19]. In contrast to latex, the major proportion of the HMG-CoA synthase from other sources is found in mitochondria [1, 19]. It is most likely that the synthase in C-serum is involved in isoprenoid synthesis (in this case largely rubber formation), like the cytosolic enzyme in other sources, whilst the enzyme in the bottom fraction may be involved in ketogenesis, like that in mitochondria.

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

Fraction	% (v/v)	Enzyme activity (%)	Specific activity (pkat mg^{-1} protein)
Rubber	51	ND	ND
C-Serum	37	70	$1\,130 \pm 40$
Frey–Wyssling	1–2	5	550 ± 50
Bottom	14	26	370 ± 90

Average value obtained from three determinations ± s.d.
ND = not determined.

Cholesterol synthesis in mammals can be regulated through the activity of both HMG-CoA reductase and HMG-CoA synthase. It was therefore of interest to study factors which might affect the activity of the synthase in C-serum. HMG-CoA synthase from various sources has usually been assayed at pH 8–8.2, suggesting that the optimum pH is within this range [1–6, 19]. The optimum pH of HMG-CoA synthase in C-serum was similar to that of the enzyme from the cytosolic fraction of rat brain [19]. The activity was *ca* 60% of the optimum at pH 6.0 or 9.5. Although the optimum pH of the enzyme is 8.2, the pH of C-serum is 6.5–7.0. This difference in pH suggests that the physiological reaction may occur in micro-environment which provides the optimum pH for the enzyme.

The rate of HMG-CoA formation (630 ± 24 pkat mg^{-1} protein, $n = 3$) did not depend on the addition of acetoacetyl-CoA up to 0.3 mM, suggesting that there is a supply of acetoacetyl-CoA in the serum. This is in good agreement with findings on the synthase in the rat brain during development [19]. On the other hand, acetoacetyl-CoA had a slight inhibitory effect on the enzyme from chicken liver [5]. In contrast, the rate of HMG-CoA formation in C-serum increased from 288 to 1 137 pkat mg^{-1} protein, when the concentration of [1-^{14}C]acetyl-CoA was raised from 0.2 to 0.8 mM. The enzyme followed Michaelis–Menten kinetics, and K_m for acetyl-CoA was 9 mM, measured at 0.05 mM acetoacetyl-CoA. The corresponding K_m for the cytosolic synthase purified from chicken liver was 100 μM, measured at 5 μM acetoacetyl-CoA [1].

The addition of 0.1 mM EDTA increased the activity of the enzyme, suggesting that the presence of endogeneous cations in the C-serum had some inhibitory effect (Table 2). The increase in enzyme activity varied from 8.5 to 28% (the average of 18% for three batches of C-serum). Addition of Mg^{2+} to various batches gave varying degrees of inhibition (Table 2). These results are possibly explained by the batches of serum having different concentrations of Mg^{2+}. In respect of these observations, the enzyme in C-serum is similar to the cytosolic synthase in rat brain [19], but differs from that in rat liver cytosol, which is activated by Mg^{2+} [5]. Mitochondrial and cytosolic enzymes from mammalian tissues have been reported to have different sensitivities to Mg^{2+}

Table 2. Effect of various substances on HMG-CoA synthase activity in C-serum of latex

	Addition	Concentration (mM)	Inhibition (% mean ± s.d.)
(a)	$MgCl_2$	5	3 ± 0.1
	$MgCl_2$	10	27 ± 10
	$CdCl_2$	0.005	52 ± 17
	$CdCl_2$	0.05	67 ± 15
	$CdCl_2$	0.1	89 ± 25
	$Pb(OAc)_2$	0.005	2 ± 0.1
	$Pb(OAc)_2$	0.05	32 ± 5
	$FeSO_4$	0.05	16 ± 0.3
(b)	$CdCl_2$	0.05	7 ± 0.7
	*p*CMB	0.5	97 ± 2
	DTT	1	4 ± 0.1
	DTT	4	34 ± 2
	DTT	8	42 ± 2

Inhibition of HMG-CoA synthase was measured by using (a) 0.1 M Tris–HCl buffer (pH 8.2) without, and (b) buffer with 0.1 mM EDTA as control. Values obtained from three determinations.

The buffered EDTA stimulated activity by 18 ± 9%.

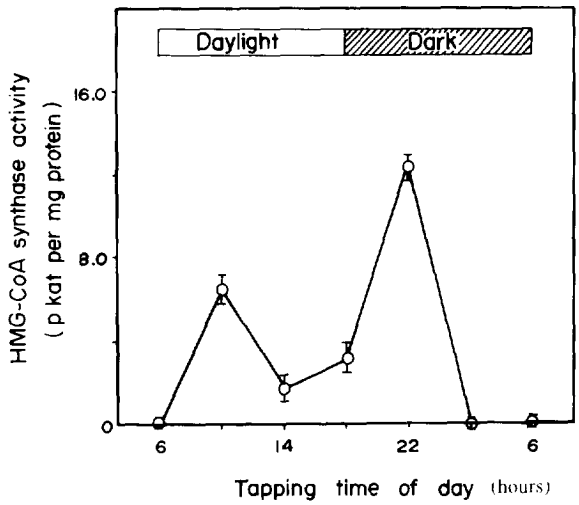

Fig. 1. Diurnal variation of HMG-CoA synthase in C-serum of latex. Each point represents the mean of 3 determinations ± s.d.

[1, 5]. The C-serum enzyme was also inhibited by Cd^{2+}, Pb^{2+} and Fe^{2+}, with Cd^{2+} being the most potent, and Fe^{2+} the least.

A sulphydryl group in avian liver HMG-CoA synthase makes this enzyme sensitive to a low concentration of Cd^{2+} (0.05 mM) [29]; this effect is reversed by EDTA. The enzyme in C-serum was also inhibited by a low concentration of Cd^{2+}, and the inhibition was reversed by EDTA (Table 2); *p*-chloromercuribenzoate (*p*CMB) also inhibited the enzyme. It is likely, therefore, that sulphydryl groups are essential to the activity of the enzyme in C-serum, although it was surprising to find that the activity was decreased by dithiothreitol (DTT), a reagent known to stabilize enzymes containing sulphydryl groups [Table 2].

The synthase was stable at − 70° for at least 70 days without major decrease in activity. The enzyme from chicken liver was also stable in frozen form [5]. Dialysis of C-serum against a 1000-fold excess of 20 mM K-Pi buffer (pH 7.0), at 4° for 24 hr, decreased the activity by 55%.

The specific activity of the synthase in C-serum fluctuated with the time the latex was tapped. The activity in the serum from clone RRIM600 was highest at 22.00 hr; i.e. 4 hr after dark (Fig. 1). Patterns of change in specific activity of the serum enzyme with tapping time, in other clones, were different from that of RRIM600 (data not shown). The times when the activity was highest in the first 24-hr cycle, and in the following cycle, were not the same. HMG-CoA synthase in rat liver cytosol also shown a diurnal rhythm, with the highest activity at 10 hr after dark [30]. Variation of activity during a 24-hr cycle has also been reported for HMG-CoA reductase in the latex of *H. brasiliensis* [31]. These findings suggest that HMG-CoA synthase may be a controlling factor in rubber synthesis, as suggested previously for the reductase [21, 25, 28].

HMG-CoA synthase, was also found in an extract of leaves, but at a much lower specific activity than in latex or C-serum (0.0–94 pkat mg^{-1} of protein, or 0–110 pkat g^{-1} in fresh leaves). Spectrophotometrically determined values for the synthase in the leaves of spinach, peas and beans were 537, 62 and 103 pkat g^{-1} in fresh leaves, respectively [22]. It was also found that the specific activity of the enzyme in leaves of different rubber clones varied (RRIM600, BP235 and BPM24 of 67, 1593 and 265 pkat g^{-1}, respectively). When the extract of leaves was fractionated into cytosol, mitochondria and chloroplasts, no activity was found in the cytosol. The enzyme was undetectable even when this fraction was further concentrated eight times by means of Amicon centriflo membrane cones CF-25 (M_r cut off 25000). Almost no activity was found in chloroplasts and mitochondria. The location of the enzyme in the leaves is at present unknown.

Though leaves contain laticifers and make rubber, the much higher specific activity (on a protein basis) of the synthase of C-serum is to be expected. The C-serum derives almost entirely from the cytoplasm of a particular cell type (the laticifer), a major function of which is to synthesize polyisoprene, whereas isoprenoid synthesis in general is a relatively minor activity of leaves.

The specific activity of the synthase in the extract made from leaves of seedlings, clone RRIM600, harvested at different times also varied (Fig. 2), as observed in C-serum.

To find out whether the synthase plays any role in the regulation of rubber synthesis, the total synthase activity and the total rubber content of 30 samples of latex, obtained by tapping the same group of four (RRIM600) trees, were determined. The two parameters correlated reasonably well (correlation coefficient + 0.81) (Fig. 3).

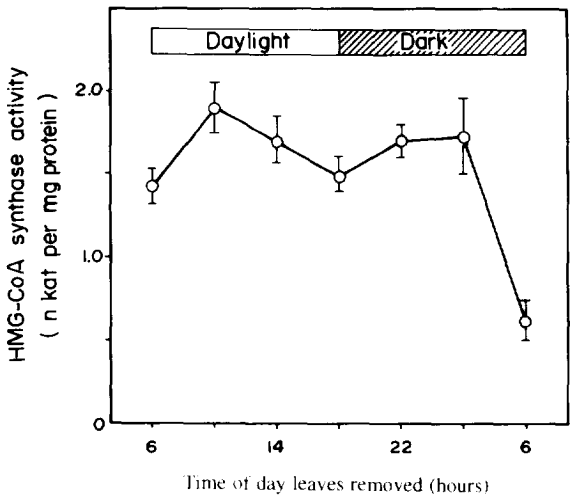

Fig. 2. Diurnal variation of HMG-CoA synthase in extracts of leaves of *Hevea* seedlings. Each point represents the mean of 3 determinations ± s.d.

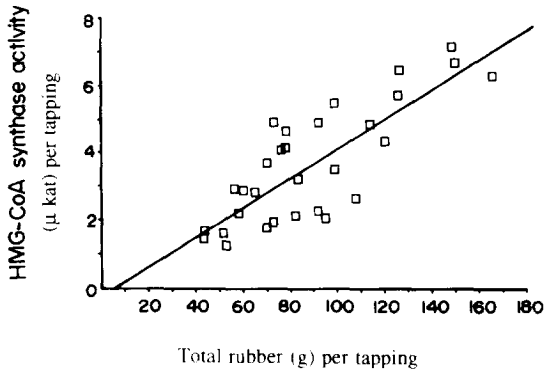

Fig. 3. Correlation between total activity of HMG-CoA synthase and dried rubber content in individual tappings of latex.

This finding is consistent with the possibility that the synthase plays some role in regulating rubber formation. On the other hand, the specific activity of the synthase decreased as the rubber content increased (correlation coefficient − 0.37). This observation is similar to the finding that cholesterol decreases the specific activity of HMG-CoA synthase and reductase in human hepatoma HepG2 cells [18].

EXPERIMENTAL

Plant materials. Fresh latex was collected (half-spiral cut, once every 2 days) from *H. brasiliensis*, clone RRIM 600, growing at the Songkla Rubber Research Centre. Generally, the trees (approximately 15 years old) were tapped at 1.00 a.m. and the latex was left in the tapping cup on ice and collected at 6.00 a.m. It was kept on ice before centrifugal fractionation in a pre-chilled rotor at 30 000 *g* (average) for 30 min. Four frs were obtained: the top rubber layer; the zone next to it containing the Frey–Wyssling particles; the clear C-serum or cytosolic fraction; the pellet (bottom fraction) containing the lutoids and other particles. C-serum was removed with a syringe; and the bottom fr. sepd by cutting the tube. All frs except rubber were assayed for HMG-CoA synthase.

For the determination of diurnal variation in the synthase activity, samples of latex were collected from the same set of trees at the time indicated in Fig. 1.

Leaves from rubber seedlings from the same clone were removed at various times of the day, washed with distilled H_2O and finely cut; the pieces were then ground in mortar with liquid N_2. Three vols of 0.1 M K-Pi, buffer (pH 7.0) containing 1 mM EDTA, 14 mM mercaptoethanol and 5% (w/v) PVPP were added to the leaves, and the suspension further homogenized twice in a Polytron (Kinematica) for 30 sec. The slurry was filtered through six layers of cheese cloth, and the filtrate assayed. In some cases, the filtrate was further fractionated into chloroplasts, mitochondria and cytosol by differential centrifugation at 3000 *g* (average) for 15 min then 30 000 *g* (average) for 45 min, and the frs assayed for the enzyme.

Chemicals for HMG-CoA synthase assay. [1-^{14}C] acetyl-CoA (2.0 GBq mmol^{-1}) and [^{14}C] HMG-CoA (0.37 MBq mmol^{-1}] were from New England Nuclear. Acetyl-CoA and acetoacetyl-CoA were from Sigma Chemical.

HMG-CoA synthase assay. HMG-CoA synthase activity was determined with a radiochemical method [1, 6, 8] using [1-^{14}C]acetyl-CoA, which was diluted with the unlabelled ester (67 dps nmol^{-1}). The reaction mixture contained 0.1 M Tris–HCl pH 8.2, 0.2 mM acetyl-CoA, 0.05 mM acetoacetyl-CoA, 0.1 mM EDTA and 10 μl enzyme sample (0–100 pkat) in a total vol. of 0.1 ml. The reaction was initiated by adding [^{14}C]acetyl-CoA to the mixture, which had been preincubated at 30° for 2 min. At 2 and 4 min after the addition, 40 μl aliquots were transferred into glass vials containing 0.1 ml 6 M HCl and dried at 95°. Water (0.5 ml) was added and the [^{14}C] HMG-CoA determined by liquid scintillation counting. To assess the amount of [^{14}C]acetyl-CoA not removed by the above procedure, a blank incubation was carried out using an assayed sample which had been boiled for 5 min.

The radioactive product from the reaction was confirmed as HMG-CoA, by PC in *n*-BuOH–HOAc–H_2O (5:2:3, v/v). The material had the same R_f as authentic [^{14}C]HMG-CoA.

Protein assay. Protein was determined as in ref. [32].

HMG-CoA lyase assay. The activity of HMG-CoA lyase was determined by the amount of [^{14}C]HMG-CoA hydrolysed after incubating [^{14}C]HMG-CoA in a reaction mixture contained 0.1 M Tris–HCl (pH 8.2) and 0.1 mM EDTA, with the samples for 15 min [19].

Rubber content of latex. Latex (10 ml) was dried at 70° for 2 days to constant wt. It is assumed that the total solid content in the latex reflects the rubber content since rubber is the major component of *Hevea* latex.

REFERENCES

1. Clinkenbeard, K. D., Reed, W. D., Mooney, R. and Lane M. D. (1975) *J. Biol. Chem.* **250**, 3117.
2. Miziorko, M. H. and Lane, M. D. (1977) *J. Biol. Chem.* **252**, 141.
3. Balasubramaniam, S., Goldstein, J. L. and Brown, M. S. (1977) *Proc. Natl Acad. Sci. U.S.A.* **74**, 1421.
4. Patel, T. B. and Clark, J. B. (1978) *Biochem. J.* **176**, 951.
5. Miziorko, H. M., Kramer, D. R. and Kulkoski, J. A. (1982) *J. Biol. Chem.* **257**, 2842.
6. Miziorko, H. M. (1985) *Meth. Enzymol.* **110**, 19.
7. Mehrabian, M., Callaway, K. A., Clarke, C. E., Tanaka, R. D., Greenspan, M., Lusis, A. J., Sparkes, R. S., Mohandas, T., Edmond, J., Fogelman, A. M. and Edwards, P. E. (1986) *J. Biol. Chem.* **261**, 16249.
8. Clinkenbeard, K. D., Sugiyama, T., Reed, W. D. and Lane, M. D. (1975) *J. Biol. Chem.* **250**, 3124.
9. Miziorko, H. M. and Behnke, C. E. (1985) *Biochem-i-stry* **24**, 3174.
10. Greenspan, M. D., Yudkovitz, J. B., Lo, C. L., Chen, J. S., Alberts A. W., Hunt, V. M., Chang, M. N., Yang, S. S., Thompson, K. L., Chiang, Y. C., Chabala, J. C., Monaghan, R. L. and Schwartz, R. L. (1987) *Proc. Natl Acad. Sci. U.S.A.* **84**, 7488.
11. Miziorko, H. M. and Behnke, C. E. (1985) *J. Biol. Chem.* **260**, 13513.
12. Meyer, R. Louis-Flamberg, P., Elliot, J. D., Fisher, M. and Leber, J. (1990) *Biochem. Biophys. Res. Commun.* **169**, 610.
13. Gil, G., Goldstein, J. L. and Brown, M. S. (1986) *J. Biol. Chem.* **261**, 3717.
14. Leonard, S., Arbogast, D., Geyer, D., Jones, C. and Sinensky, M. (1986) *Proc. Natl Acad. Sci. U.S.A.* **83**, 2187.
15. Popplewell, P. Y. and Ahzar, S. (1987) *Endocrinology*, **121**, 64.
16. Salam, W. H., Wilcox, H. G., Cagen, H. G. and Heimberg, M. (1989) *Biochem J.* **258**, 563.
17. Smith, J. R., Osborne, T. F., Slaughter, C. A., Brown, M. S., Goldstein, J. and Gil, G. L. (1988) *J. Biol. Chem.* **263**, 18480.
18. Rosser, D. S. E., Ashby, M. N., Ellis, J. L. and Edwards, P. A. (1989) *J. Biol. Chem.* **264**, 12653.
19. Shah, S. N. (1982) *Neurochem. Res.* **7**, 1359.
20. Servouse, M. and Karst, F. (1986) *Biochem. J.* **240**, 541.
21. Lynen, F. (1969) *J. Rubb. Res. Inst. Malaya* **21**, 389.
22. Alam, A., Britton, G., Powls, R. and Goach, J. (1991) *Biochem. Soc. Trans.* **19**, 164S.
23. Bach, T. J., Boronat, A., Caellees, C., Ferrer, A., Weber, T. and Wettstein, A. (1991) *Lipids* **26**, 637.
24. Wititsuwannakul, R., Wititsuwannakul, D., Sothibandhu, R., Suvachittanont, W. and Sukonrat, W. (1988) *Proceedings of the IRRDB Rubber Physiology and Exploitation Meeting*, Paris, p.161.
25. Sipat, A. B. (1982) *Phytochemistry* **21**, 2613.
26. Wititsuwannakul, R., Wititsuwannakul, D. and Suwanmanee, P. (1990) *Phytochemistry* **29**, 1401.
27. Chye, M.-L., Tan, C.-T. and Chua, N.-H. (1992) *Plant Mol. Biol.* **19**, 473.
28. Hepper, C. M. and Audley B. G. (1969) *Biochem J.* **114**, 379.
29. Miziorko, H. M., Behnke, C. E. and Wang, H.- H. L. (1990) *Biochim. Biophys. Acta* **1041**, 273.
30. Royo, T., Ayte, J., Alberico, F., Giralt, E., Haro, D. and Hergadt, F. G. (1991) *Biochem J.* **280**, 61.
31. Witisuwannakul, R. (1986) *Experientia* **42**, 44.
32. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.