PROGRESS REPORT NO.5

"BIOTECHNOLOGY APPLICATION FOR CHARACTERIZATION AND SELECTION OF BETTER-YIELDING RUBBER CLONES"

A RESEARCH PROJECT

USAID/PSTC PROGRAM

Grant No. 936-5542-G-00-6054-00

Submitted by

Dr. Rapepun Wititsuwannakul

Project Leader

Department of Biochemistry

Faculty of Science

Prince of Songkla University

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Project Profile

Country	: Thailand
Grant No.	: 936-5542-G-00-6054-00
Program	: Program on Science and Technology Cooperation
Project Titl	: Biotechnology Application for Characterization and Selection of Better-Yielding Rubber Clones.
Project Leader	: Dr. Rapepun Wititsuwannakul
Organization	: Dept. of Biochemistry, Faculty of Science, Prince of Songkla University, Hat-Yai 90112, Thailand.
Co-investigstors	: Dr. Wallie Suvachitanont, Dr. Papiporn Sotthibandhu, Dr. Dhirayos Wititsuwannakul, Mr. Apichai Shuprisha, Mr. Wisut Sukonrat, Dr.Nanta Churngchow
Authorized Officer	: Dr. Phasook Kullavanij (Rector)
Total Project Budget	: US $ 150,000
Project Duration	: 3 years (27 June-1986-31 July 1989)
Reporting Period	: 1 July-31 December 1988
Budget Allocation for This Period	: US $ 119,669.

1. Background/Introductions :

Part A. Biochemical parameters for rubber yield evaluation in *Hevea brasiliensis*.

Traditional practice on yield evaluation of rubber is to grow the plant for at least six years until trees can be tapped and yield measured. Early attempt on yield evaluation studying relation between latex yield of *Hevea* and rubber biosynthesis in *vitro* was reported in 1965 by D'Auzac (1). The study involved latex specimens chosen from pairs of high-and low-yielding trees growing in a monoclonal plot. The efficiency of rubber biosynthesis from 2-^{14}C acetete was shown distinctlyl higher in the high yielders. In 1970, C.H. Woo and E.E. Edwin performed similar incorporation experiment using trees from different clones and chosen at random (2). The results led to similar conclusion but large variations made it difficult to make reliable distinction of high low-yielding potentials. Our studies on diurnal variation of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) activity showed the enzyme was closely related to rubber content in the latex (3). The correlation suggested possible role of this enzyme in regulation of rubber biosynthesis. In this study we proposed to test and verify HMGR as a rate limiting enzyme in rubber biosynthesis pathway. Its possible use as an enzyme parameter in yield evaluation will be further pursued and utilized in clonal selection processes.

Part B. Disease resistance screening of *Hevea* at tissue cluture stage

Since *Hevea brasiliensis* is a perennial tree crops and thus the duration of breeding cycle is rather long. It takes 5-6 years for *Hevea*

to grow before the first tapping can be commenced, after which it can be tapped for rubber latex for a period of 30 or more years. It is, therefore, of great importance to select for a disease resistant clones in addition to the high-yielding potential concurrently. This is an added advantage to prevent growth and rubber production failure due to disease or disturbance by the pathogens. We proposed a system to study the disease resistance at tissue culture level in this study as an early selection in conjunction with high-yielding potentials.

2. Objectives :

A. To develop a reliable method for identification and selection of higher yielding rubber clones at an early stage, while the plants are still at seedling stage. This would help circumventing the time and energy needed in conventional approaches.

B. To initiate the study of disease resistant rubber clones at the level of tissue culture stage in conjunction to the high-yielding potential screening as outlined below.

Yield screening at seedling stage (Part A)

Potential low yielder (discard) | Potential high yielder

Disease resistance screening at tissue culture stage (Part B)

susceptible clone | resistant clone

Adopted as possible high yielding disease resistant clones.

3. Materials and methods :

Part A. Biochemical parameter for rubber yield evaluation in *Hevea brasiliensis*.

Chemicals:

All chemicals and reagents used were of analytical grade. DTT NADPH, HMG-CoA, polyoxyethylene ether W-1 (Brij W-1), mevalonolactone, chicken egg albumin, carbonic anhydrase, urease (trimer, hexamer) and bovine perum albumin (monomer, dimer) were from Sigma. $3^{14}C$ HMG-CoA was from Amersham England and Affi-Gel-Blue was from Bio-Rad. Agarose-hexane-HMG-CoA was from P-L Biochemicals.

Collection and fractionation of latex :

Latex was obtained from several clones of *Hevea brasiliensis* as indicated in the text. The trees are reqularly tapped, half spirally on every other day (S.2/D.2). All clones are grown categorized at the Rubber Research Center, Hat-Yai. Rubber trees were normally tapped at 0600 h or otherwise as indicated. After tapping the fresh latex was collected into an ice-chilled beaker. The latex from each clone was pooled and fractionated into 3 major fractions by centrifugation in a microfuge at 15,000 rpm for 60 min. They are the rubber fraction, the C-serum (latex cytoplasm) and the bottom fractions, respectively. The bottom fractions, where 95% of HMGR was located (4), were separated by cutting the bottom of the microtube and used for assay of HMGR activity.

Assay of HMG-CoA reductase (HMGR) activity :

The HMGR activity was assayed in buffer x (0.1 M KCl, 10 mM DTT 30 mM EDTA, 0.1 m K_xPO_4, pH 7.0) containing bottom fraction or active protein

fraction or fresh latex with protein ranged from 0.1-1.8 mg, 0.4 µmoles NADPH, 120 nmoles DL-3 ^{14}C HMG-CoA (1000 dpm/nmole) in a final volume of 50-100 µl. For solubilized enzyme, 1% W/V of BSA was added to the assay system to stabllize the HMGR activity. Incubation was at 37°C for 20 or 30 min as indicated. The reaction was stopped by addition of 25 µl of 10 N HCl. Unlabeled mevalonolactone was then added, and the mixture was allowed to incubate for another 30 min for complete lactonization of the incubation product (mevalonic acid). The precipitated protein was removed by centrifugation and the product of the enzyme was purified by TLC using benzene acetone (1:1 v/v) as solvent system and developed under iodine vapor according to the method of Iijima *et al.* (5). The zone of mevalonolactone was scraped into a scintillation vial containing 10 ml of dioxane fluor. Samples were allowed to stabilize overnight before counting in Packard liquid scintillation counter.

Enzyme solubilization and fractionation : Frozen sample of bottom fraction was thawed at room temperature and homogenized in equal volume of solubilization buffer A (0.1 M K_xPO_4, pH 7.0, 10 mM DTT 0.1 m KCl, 5 mM EDTA) containing 1% Brij W-1 and 20% glycerol. The detergent Brij W-1 was used according to method described by Bach *et al.* (6). A glass Potter Elevehjem homogenizer with a tight-fitting glass pestle was used in homogenization. After 10 downward passes of hand-driven glass homogenizer pestle, the suspension was incubated at 37°C for 30 min. The mixture was then rehomogenized and centrifuged at 190,000 g for 60 min at 4°C. The resulting supernatant was isolated and fractionated by slow addition of saturated ammonium sulfate solution to final concentration of 40%. After incubation

at $4^{o}C$ temperture for 3-6 hours the mixture was centrifuged at 25,000 g for 30 min. The yellowish uppermost protein layer was dissolved in buffer B containing 6% Brij W-1 and dialyzed against buffer B (50 mM K_xPO_4,5 mM EDTA, 0.1% Brij W-1, 10 mM 2-Mecaptoethanol) before subjected to further purification by chromatography.

Chromatographic procedures :

The dialized sample in buffer B was slowly loaded onto a column (40 x 2.5 cm) of DEAE Sephadex A-50, previously equilibrated with buffer B, and washed with buffer B with flow rate 0.5 ml/min. The enzyme protein was eluted by adding KCl in buffer B at concentration 50, 500 and 1000 mM respectively. The enzyme fractions were pooled and concentrated by ammonium sulfate precipitation (90% saturation). The pellet was collect by centrifugation at 25,000 xg for 30 min and dialyzed against buffer B. This active fraction was then loaded on Affi-Gel-Blue (100-200 mesh; 75-150 μ) column (1.5 x 5 cm),previously equilibrated with buffer B. The column was washed with buffer B before eluted with the same buffer by the addition of 500 mM and 1000 mM KCl respectively. Active fraction were pooled and loaded onto 1 ml HMG-CoA-hexane agarose column, previously equilibrated with buffer B. The enzyme activity was eluted by adding 100 mM KCl, 100 mM KCl + 5 mM NADPH mixture, 750 mM KCl and 1000 mM KCl respectively.

Molecular weight determination of HMGR :

HMGR molecular weight determination was performed by methods described in Sigma Tech. Bulletin No.MRK-137 (10-86)

Gel electrophoresis :

Polyacrylamide gel electrophoresis (PAGE) was run either in the absence of sodium dodecyl sulfate (SDS) using method of Weber et al. (7). The method of Wray et al. (8) was used for silver staining.

Protein derermination :

The protein concentration was determined by the method of Lowry et al. (9).

Preparation of antibody against HMGR :

Rabbit immunization

Approximately 50 µg of purified HMGR in 0.5 ml of complete Frend's adjuvant was prepared for each injection dose. The protein antigen mixture was injected subcutaneously to rabbits at 1 week interval. The titer of antidy was tested at least 4 days after the third injection.

Immunotitration of enzyme

Antibody against HMGR was test for specificity using Ouchterlony immunodifusion against purified as well as enzyme in fresh latex, crude extracts of leaf and callus. Various amount serum from immunized rabbit was incubated with purified HMGR in fresh latex to test for its ability to inhibit the HMGR activity, using preimmunized rabbit serum as a control.

Part B. Disease resistance screening of *Hevea* at tissue culture stage.

Explant materials

Hevea flowers obtained from late flowering season. Pistillate flowers located at the top of central axis ans main lateral branches were

collected. Male flower buds (3-3.5 mm) in which majority of the anthers are at uninucleate stage were excised and used for callusing in various types of culture media as indicated.

Very young *Hevea* seeds with diameter zise of 1.5 cm was used to prepare or isolate the inner *Hevea brasiliensis* seed tegument. An outer greenish skin of seeds was peeled and inner seed tegument was prepared by using sterile blade. Thin longitudinal radial slices (1 mm thick) of inner seed tegument were cut and placed in MB culture medium. The tissue were incubate at room temperature with 12 hrs. of dark-light cylce.

Medium preparation for *Hevea* tissue culture

1. MB medium

macronutrients

KNO_3	954	mg/l
NH_4NO_3	650	mg/l
$MgSO_4.2H_2O$	370	mg/l
$CaCl_2$	530	mg/l
KH_2PO_4	503	mg/l

micronutrients

H_3BO_3	1747	mg/l
$MnSO_4.4H_2O$	1003	mg/l
$ZnSO_4.7H_2O$	8325	mg/l
$Na_2MoO_4.5H_3O$	241.9	mg/l
$CuSO_4.5H_2O$	249	mg.l

Iron Salts

Na_2-EDTA	40.9	mg/l
$FeSO_4.7H_2O$	30.5	mg/l

Organic gr. Substance

Nicotinic acid	492.4	mg/l
Glycine	2252.1	mg/l

Phytopthora botryosa culturing :

Phytopthora botryosa was obtained from Plant Disease Division, Songkla Rubber Research Center. The fugus was subcultured once every two weeks in potato dextrose agar (PDA) solid medium containing (per liter) 3.9 gm PDA and 0.02 gm lecithin. P-4 broth medium containing (per liter) 0.5 gm KH_2PO_4, 0.5 gm K_2HPO_4, 0.5 gm $MgSO_4.7H_2O$, 0.02 gm $CaCl_2.2H_2O$, 1 gm thiamine, 20 gm glucose, 1.5 gm $NaNO_3$ and 0.2 gm lecithin was used for suspension culture.

Extraction of Phytophthora toxin

An agar disc (5 mm diameter) containing phytophothora was prepared by cutting around the margin area of 7-10 days old phytopthora colony grown on PDA solid medium. It was then cultured into 500 ml of suspension culture medium and incubated for 7 days. Approximately 4500 spores/ml was obtained from a week old culture. The culture fluid was prepared by filtration through three layers of cheesecloth. The culture filtrate was concentrated at 60°C to 10% of its original volume. An equal volume of methanol was added and solution was stored overnight at 5°C. Precipitate obtained after the

methanol addition was removed by filtration through Whatman no.1 filter paper. Mathanol was evaporated at 60°C and the water fraction was partitioned with three volumes of chloroform. The chlorofrom extract was evaporated in the hood and the remaining oily substance was weighed and dissolved in small quantities of methanol. The volume was adjusted to 2% of the original volume of the culture filtrate. The final concentration of extracted toxin was 3000 µg/ml. The toxin with final concentrations of 2.5, 25, 250, 275, 300 µg/ml in 4.5 ml MB medium were prepared and used to culture 45 days old callus initiated from RRIM 600 and GT 1 male flowers.

4. Results/Discussions/Tables :

Part A. Biochemical parameter for evaluation of rubber yield in *Hevea brasiliensis*.

A.2 Solubilization, Purification and Characterization of HMGR

Preparation of soluble HMGR : Various attempts to solubilize HMGR from the bottom fraction of fresh latex were carried out using different detergent compounds. Efforts to solubilize the enzyme with Trition x-100, Brij 35 and Tween 80 were unsatisfactory. However, we found that maximum activity can be obtained in soluble form by using Brij (polyethylene ether) W-1 under conditions similar to those reported for etiolated radish seedlings by Bach et al. (6). After the solubilization process, all enzyme activity was present in the soluble or supernatant fraction. The enzyme activity remained stable in this solubilization medium.

Purification of solubilized reductase enzyme : Basically the protocol employed was recently developed for the purification of radish HMGR (6) with some modifications. The concentration and purification of HMGR

was achieved by ammonium sulfate fractionation and affinity chromatography. Affi-Gel-Blue, a biospecific affinity medium for purifiying nucleotide requiring enzyme and agarose-hexane-HMG-CoA were used in the final affinity chromatography step. Data for a typical preparation and purification are presented in Table I. The specific activity of the reductase enzyme solubilized from a freshly prepared bottom fraction ranges between 0.4 to 0.7 nmole $min^{-1}mg^{-1}$. Solubilization leads to a 1.78 fold purification so that the specific activity of the enzyme in the soluble supernatant after centrifugation is around 0.7 nmol $min^{-1}mg^{-1}$. Further purification was achieved by fractionating the supernatant with addition of saturated ammonium sulfate solution to final concentration of 40%. The uppermost yellowish enzyme protein layer was collected by centrifugation at 25,000 g for 20 min. The ammonium sulfate fractionation resulted in about 5 fold purification of the enzyme. The enzyme was further purified by chromatography on DEAE-Sephadex A-50. The elution profile obtained is shown in Fig. 1. The reductase activity was eluted from the column with buffer B containing 0.5 M KCl. The pooled fraction contained approximately 85% of the added activity and gave 22 fold increase in specific activity. Further purification on Affi-Gel-Blue (Fig. 2.) and HMG-CoA-hexane agarose columns (Fig.3) led to 77 and 257-fold of purification respectively. Polyacrylamide gel electrophoresis (in the presence of SDS) of 10 µg of protein from the purified enzyme was carried out. The enzyme with a specific activity of 105 nmol $min^{1}mg^{-1}$ was applied to the gel and exhibited a single major protein band (Fig.4.). The calibrate subunit molecular weight or *Hevea* HMGR was approximate 44,000 daltons(Fig.5.)

The subunit molecular weight of HMGR from *Hevea* was similar to that reported for radish seeding (6) and considerably lower than that reported for the intact liver enzyme as deduced from nucleotide sequence by liscum et al. (10,11). The purified enzyme showed a single prominant band with molecular mass of 176,000 daltons in the nondenaturing gel electrophoresis as visualized by silver staining procedure (Fig.6,7). This value favorably corresponds to the size of 180,000 daltons obtained for the radish enzyme using density gradient centrifugation (6). However, some proteolytic degradation during the solubilization and purification may occur. The value for the Michaelis-Menten constant of 13.3 u M with respect to (S)-HMG-CoA was observed with purified *Hevea* HMGR (Fig.8). The Km value is higher than purified yeast, radish seedlings, rat and chicken liver enzymes (6, 12) and the range of Km reported for *Fusarium oxyosporum* enzyme (12). It is, however, quite lower than Km value of 56 uM which was reported for HMGR of the crude membrane fraction obtained from the bottom fraction of latex (13).

The stability of HMGR is generally dependent upon the presence of a reduced thiol compound (12). Early studies demonstrated that HMGR in bottom fraction was activated by dithiothreitol with an optimal concentration at 2 mM (4). Our present study reveals that a higher concentration of dithrothreitol (20 mM) is necessary to maximize catalytic activity of purified HMGR (Fig.9).

The pH allowing maximal activity for all HMGR assays analyzed thus far is approximately 7.0 ranging from 6.5 to 7.7 (14, 15). An optimum pH of 6.8-7.0 was reported for *Hevea* HMGR in the bottom fraction suspensions(4).

We found an optimum pH of 7.0 for the purified HMGR which is similar to that reported for HMGR (Fig.10).

A.2 Preparation of antibody against HMGR

The antibody was obtained from immunized rabbit serum after one week following the third injection. The titer of rabbit antiserum is very low (1:1). The antiserum was able to pracipitate HMGR in fresh latex, bottom fraction or purified HMG-CoA reductase from. Single precipitin band was observed from double gel diffusion. Purification of antiserum IgG and linking of purified IgG to alkaline phosphatase will be performed in order to develop ELISA for the HMG-CoA reductase measurement. The test for the ability of HMGR antibody to inhibit HMGR activity is still in progress.

A.3 Preliminary yield screening on new *Hevea* germplasm

Second attempt on additional preliminary yield screening on 55 new *Hevea* germplasms were made on *Hevea* leaf latex measurements. This was carried out by evaluating the yield on rubber latex upon cutting through leaflet of 3 months old seedlings using method of Zhongyu *et al.*(16). Leaf blade was cut parallel on both sides of midrib vein with 1 cm apart. Amount of latex being excreted from lateral veins were then measured. Grades A to D were given for high to low latex yield obtained. Results on yield evaluation by estimating latex excreted from lateral veins are shown in Table II. There were only 7 out of 55 new *Hevea* germplasms which demonstrate high yield potential. They are subjected to further yield evaluations by comparing their latex HMGR level obtained by making microtapping on the stem and compared with

RRIM 600 latex under the same condition and treatment. Results on latex HMGR levels on RRIM 600 and the 7 new *Hevea* germplasms are shown in Table III. Most of HMGR activity levels on 7 new *Hevea* germplasms test are lower than that of RRIM 600 which is the established known high yielder. There is only one new clonal type from MT/IT/12 type of material source under 26/34 coding that demostrated higher latex HMGR activity than that of the RRIM 600 clone. Presently we obtained 2 potential high yielders from the screening of 215 new *Hevea* germplasms (see progress report no.3 for previous screening). More extensive screening is underway.

Part B. Disease resistance screening of *Hevea* at tissue culture stage

B.1 Callus culture from *Hevea* inner seed tegument

Callus formation from inner seed tegument were obtained (Fig.11a, 11b) after a few weeks of culturing. Faster growing callus was observed from the explants of inner seed tegument than that of male flowers. This callus will be subcultured and used as source of tissue sample for HMGR measurement by the ELISA technique.

B.2 Effect of phytophthora toxin on callus growth

From our previous studies (progress report no.4) on disease resistance assessment using life pathogen infection on young *Hevea* leaves, we were able to show that the leaf of GT-1 is more resistant to phytophthora infection than that of RRIM 600. In this report, various concentrations of phytophthora toxin extract were added to the MB culture media and used to culture 45 days old calli initiated from male flowers of GT-1 and RRIM 600

clones. It is shown in Table IV that after one week of toxin innoculations toxin at 25 ug/ml was not able to kill any type of calli whereas toxin at 275 ug was able to completely kill RRIM 600 callus. Toxin at 250 ug/ml however, was able to killed only 33.3% of total GT-1 calli. This result indicates that IIRM 600 callus is more susceptible to phytophthora toxin than GT-1 callus and the expression on disease resistance may be detectable at callus level. Similar test on the survival rate on callus initiation of GT-1 and RRIM 600 male flowers in media containing toxin was revealed in Table V. It was seen that after the sixth week of flower innoculations, almost all RRIM 600 calli were dead while GT-1 calli were still alive with mormal growth. Moreover, the growth rate of RRIM 600 calli before cessations were very poor when compared to its control.

5. Conclusion remarks

-The molecular mass of HMGR estimated from non-denaturing gel electophoresis is 176,000 dlatons with subunit molecular weight under SDS-PAGE of 44,000 daltons.

-The optimal pH of HMGR activity is around 7 with an apparent Km of 13.3 uM for (S)-HMG-CoA.

-A low concentration of dithiothreitol is still required to maximize catalytic activity of the purified enzyme.

-Antobody against HMGR was obtained.

-Callus could be initiated from *Hevea* inner seed tegument.

-Callus initiated from GT-1 male flower was more resistant to phytophthora toxin extract in culture media than that of RRIM 600

-Callus initiation in media contiaing phytophthora toxin was not successful with RRIM 600 male flowers but successful with that of GT-1

6. Work plan for next period

-Develop ELISA technique for HMGR measurement.

-Studies on diurnal variation of MVA kinase and HMG-CoA synthetase in *Hevea* leaves.

-Continuation studies on yield evaluations of new *Hevea* germplasms.

7. References :

1. J.D' Auzac (1965) Relations entre la composition chimique du latex, 1' intensite de quelques reaction metaboliques et la productivite de 1' *Hevea brasiliensis*. Revue gen. Caoutch, Plastiq. *42(78)*, 1027.

2. C.H. Woo and E.E. Edwin (1970) Relationship between yield of and rubber biosynthesis *in vitro*. J. Rubb. Res. Inst. Malaya *23*, 68.

3. R. Wititsuwannakul (1986) Diurnal variation of 3-hydorxy-3-methyl-glutaryl coencyme A reductase activity in latex of *Hevea brasiliensis* and its relation to rubber content. Experientia. *42*, 44.

4. A.B. Sipat (1982) Hydroxymethylgltaryl CoA reductase (HADPH) in the latex of *Hevea brasiliensis*. Phytochemistry *21*, 2613.

5. Y. Iijima and M.Maryyama (1873) Simplification of the assay of 3-hydroxy-3-methylglutaryl CoA reductase activity. 15th. Proceedings of Japan Council of Biochemistry of Lipids *15*, 164.

6. J.J. Bach, D.H. Rogers and H.Rudney (1986) Detergent-solubization, purification, and characterization of membrane-bound 3-hydroxy-3-methylglutaryl-coenzyme A reductase from radish seedlings. Eur. J. Biochem. *154* , 103.

7. K, Weber and M. Osborn (1969) the reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. *244* , 4406.

8. W. Wray, T. Boulikas, V.P. Wray and R. Handcock (1981) Silver staining of proteins in polyacrydamide gels. Anal Biochem. *118* , 197.

9. O.H. Lowry, N.J. Rosenbrough, A.L. Farr and R.J. Randall (1951) Protein measurement with Folin phenol reagent. J. Biol. Chem. *193* , 265.

10. L. Liscum, K.L. Luskey, D.L. Chin, Y.K. Ho, J.L. Goldstein and M.S.Brown (1983) Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase and its mRNA in rat liver as studied with monoclonal antibody and a cDNA probe. J. Biol. Chem. *258* , 8450.

11. L. Liscum, R.D. Cummings, R.G.W. Anderson, G.N. De Martino, M.S. Brown (1983) 3-Hydroxy-3-methylglutaryl-CoA reductase : a transmembrane glycoprotein of the endoplasmic reticulum with N-linked "high mannose" oligosaccharides. Proc. Natl. Acad. Sci. U.S.A. *80* , 7165.

12. H.D. Rogers, S.R. Panini and H. Rudney (1981) in 3-hydroxy-3-methylglutaryl coenzyme A reductase, ed. R. Sabine, CRC Press, Florida.

13. A.B. Sipat (1982) Arrhenius plot characteristics of membrane bound 3-hydroxy-3-methylglutaryl coenzyme A reductase from the latex *Hevea brasiliensis*. Biochem. Biophys. Acta *705(2)*, 248.

14. W.E. Brown and V.W.Rodwell (1980) in Dehydrogenases requiring nictinamide coenzymes, ed. J. Jeffery, Birkhauser Verlay, Basel.

15. N. Qureshi and J.W. Porter (1981) in Biosynthesis of isoprenoid compounds, vol. 1, eds. J.W. Porter and S.L. Spurgeon. John Wiley & Sons, New York.

16. Z. Zhongyu, Y. Xiehui, W. Lizhen, G. Qiyuan, H. Xiang, Z. Sarirong, C. Chuangin and L. Jinxing (1984) Approaches to early prediciting mature rubber yield potential at juvenile stage and their theoretical basis, paper from South China Academy of Tropical Crops, presented IRRDB Conference, Beijing, China.

8. Annex Picture :

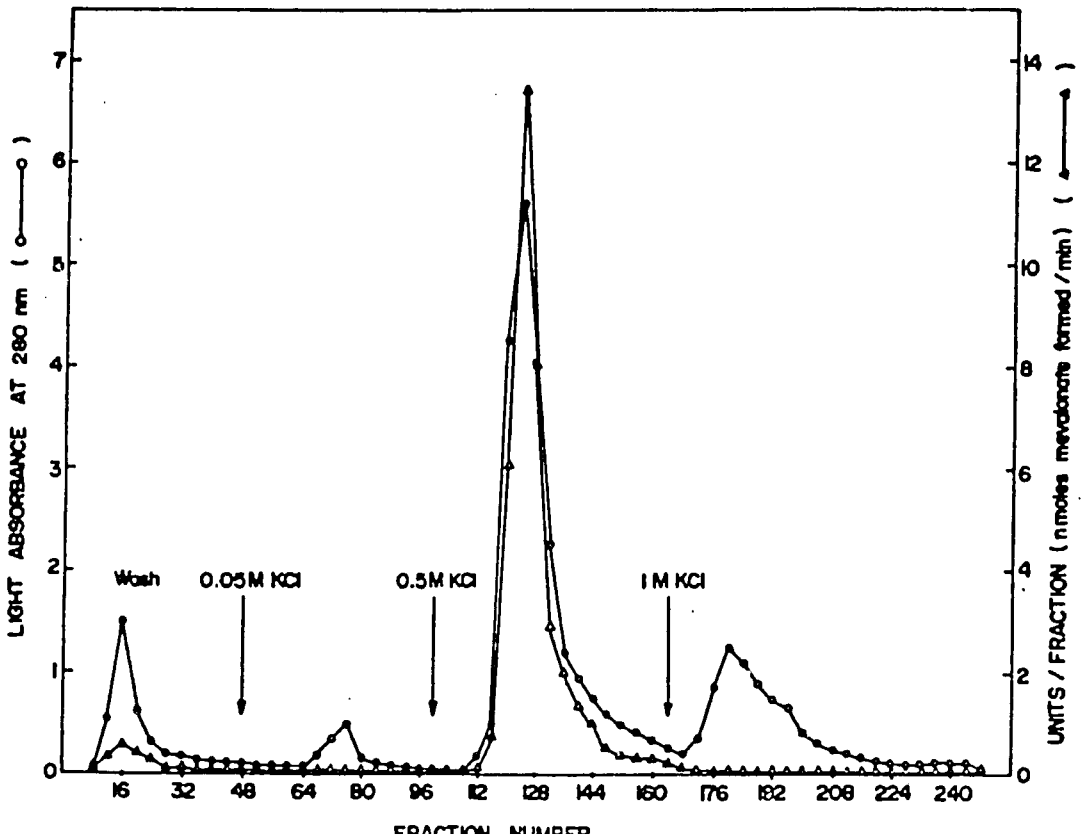

Fig. 1. Purification of detergent-solubilized HMGR on DEAE-Sephadex A 50. Position of arrows, indicating the change of salt concentrations.

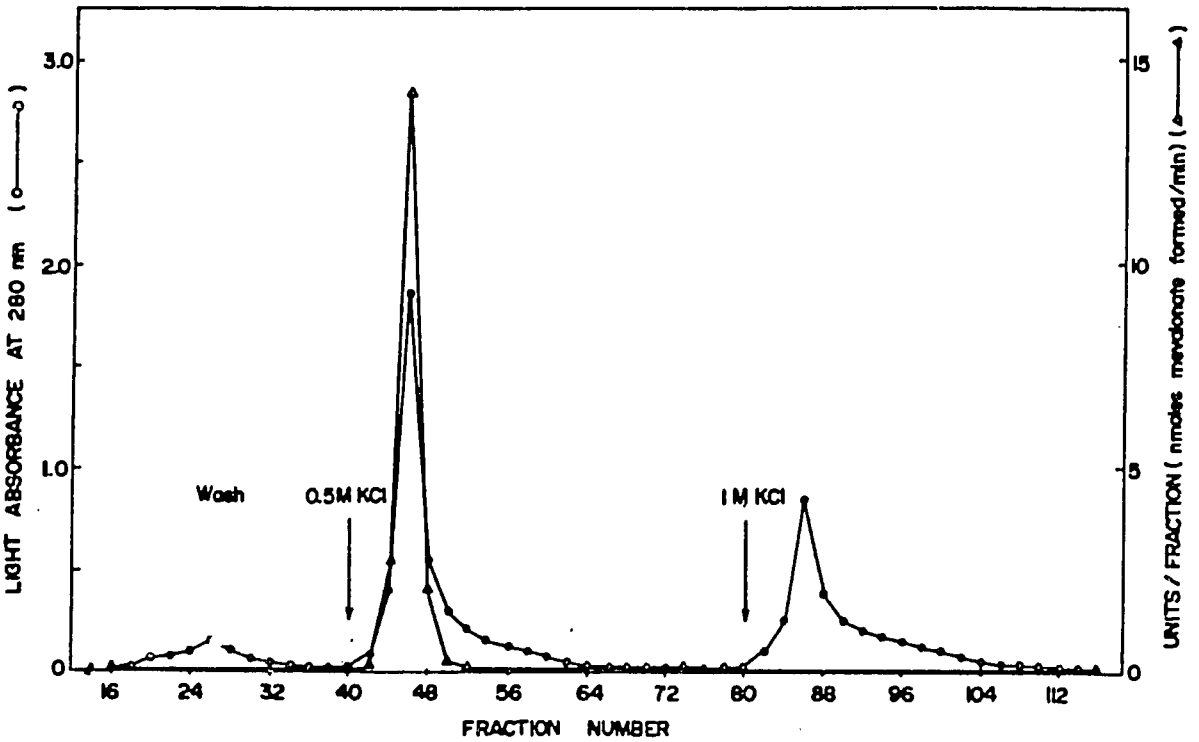

Fig.2. Purification of active fractions obtained from the DEAE-Sephadex column on Affi-Gel Blue column. Position of arrows indicating changes of salt concentrations.

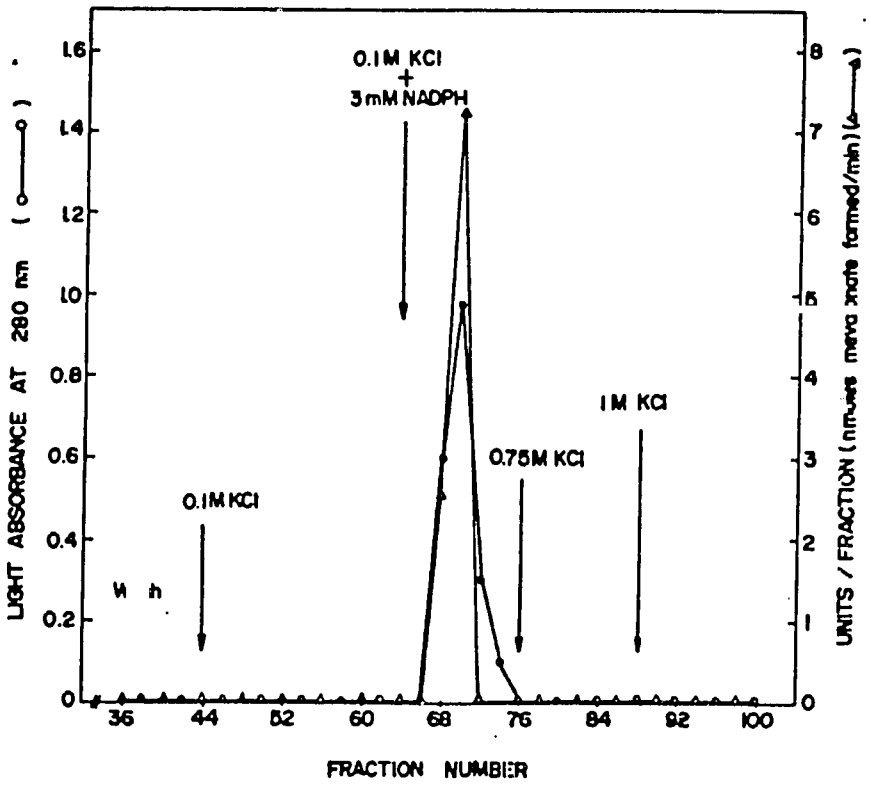

Fig. 3. Purification of HMGR on HMG-CoA-hexane-agarose. Arrows indicate change of salt concentrations with or without NADPH addition.

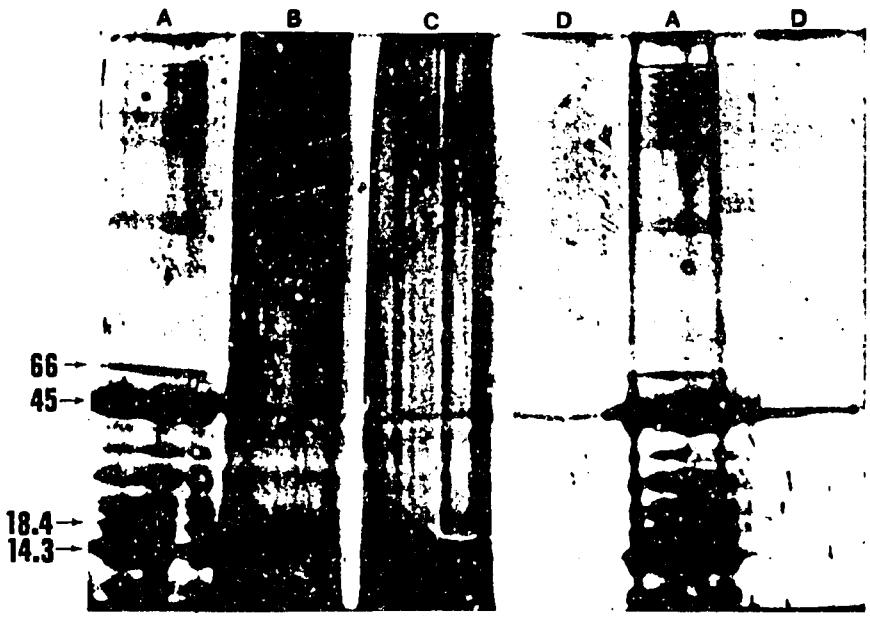

Fig. 6 Non-denaturing gel electrophoresis of purified HMGR obtained after HMG-CoA hexane agarose column. The relative positions of protein standards are as indicated in lane A (K Da). Lanes B,C and D represent peak fraction proteins from DEAE Sephadex, Affi-Gel Blue and HMG-CoA-hexane agarose columns, respectively.

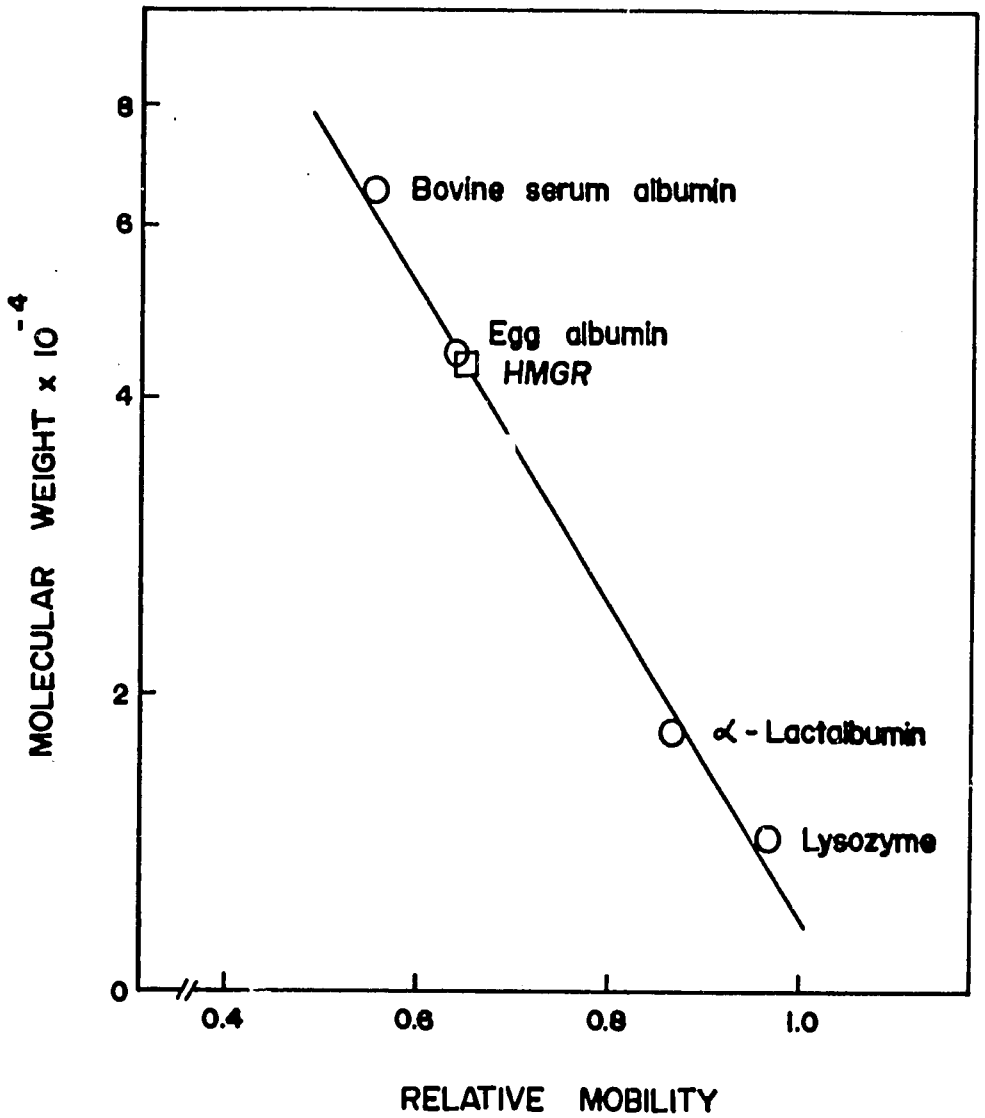

Fig. 5 Molecular weight calibration of HMGR monomeric polypeptide. Both purified enzyme and standard proteins were performed on the same slab gel under 8% SDS-PAGE.

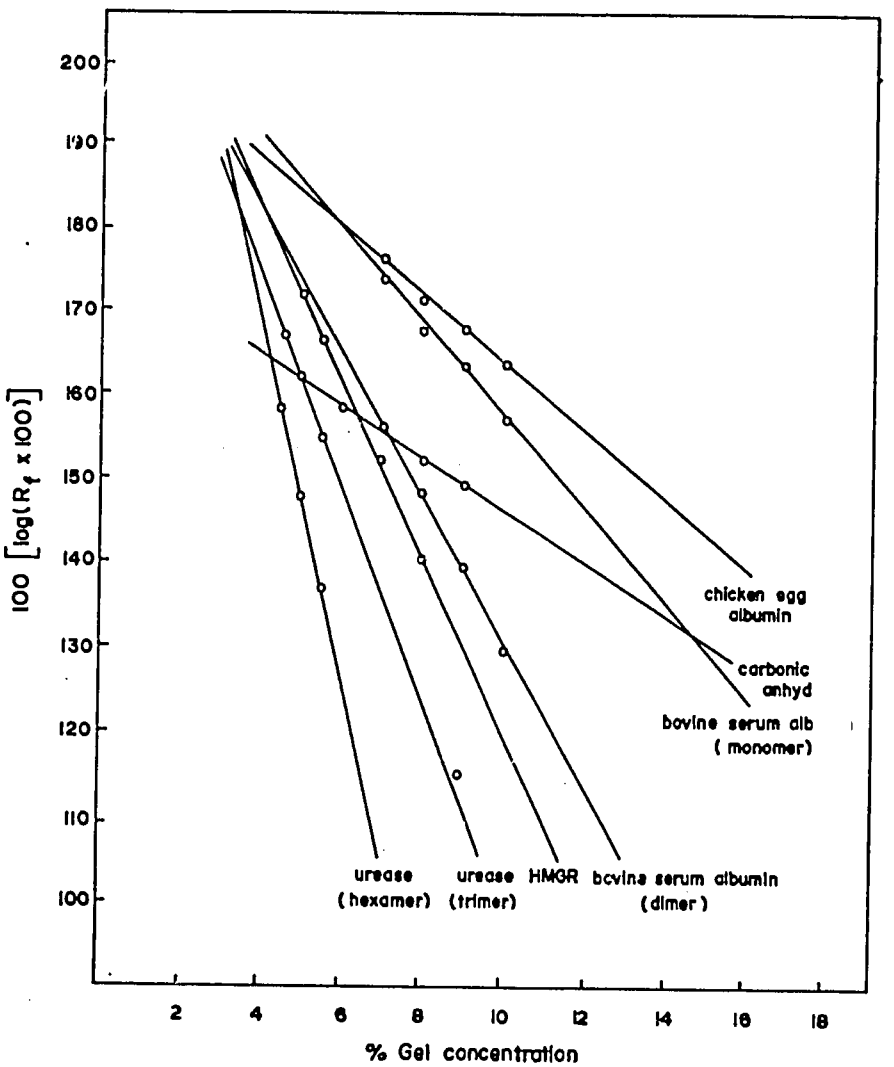

Fig. 6 Determination of retardation coefficient (slope) of standard proteins for HMGR molecular weight calibration in non-denaturing gel electrophoresis as described in Sigma Tech. Bulletin No. MRK-137 (10-86).

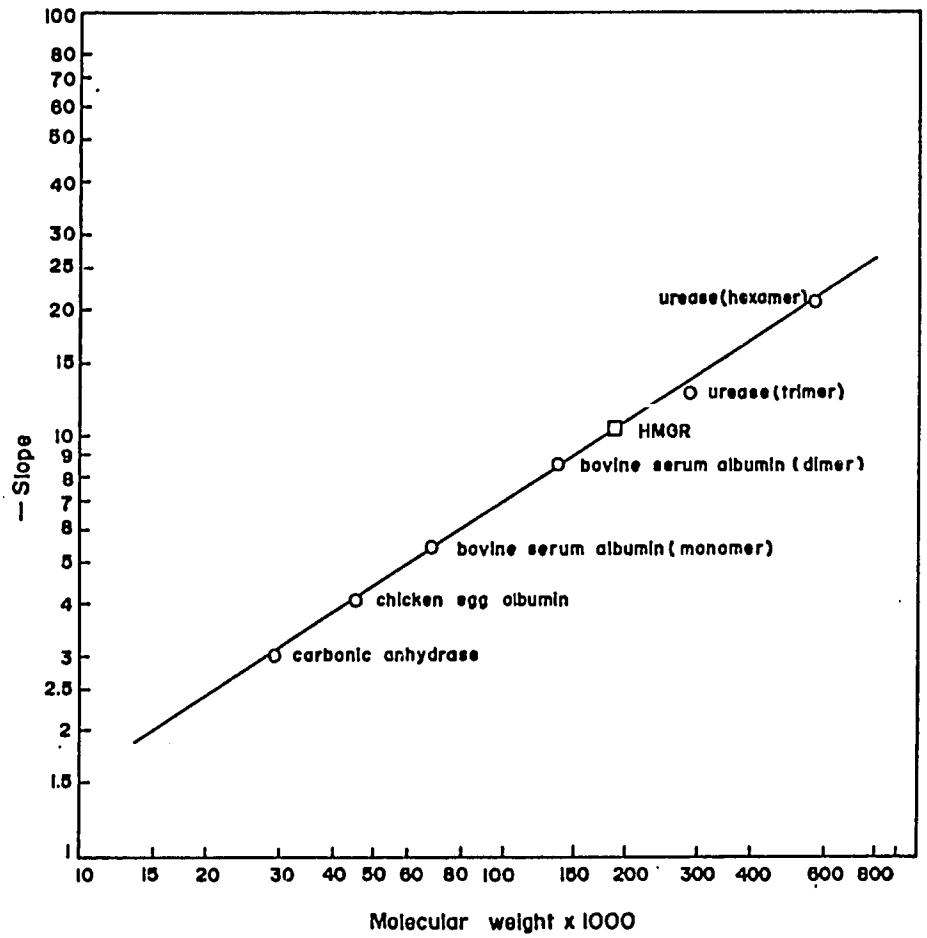

Fig. 7 Molecular weight calibration of HMGR in a non-denaturing gel electrophoresis performed according to method described in Sigma Tech. Bulletin No. MRK-137 (10-86).

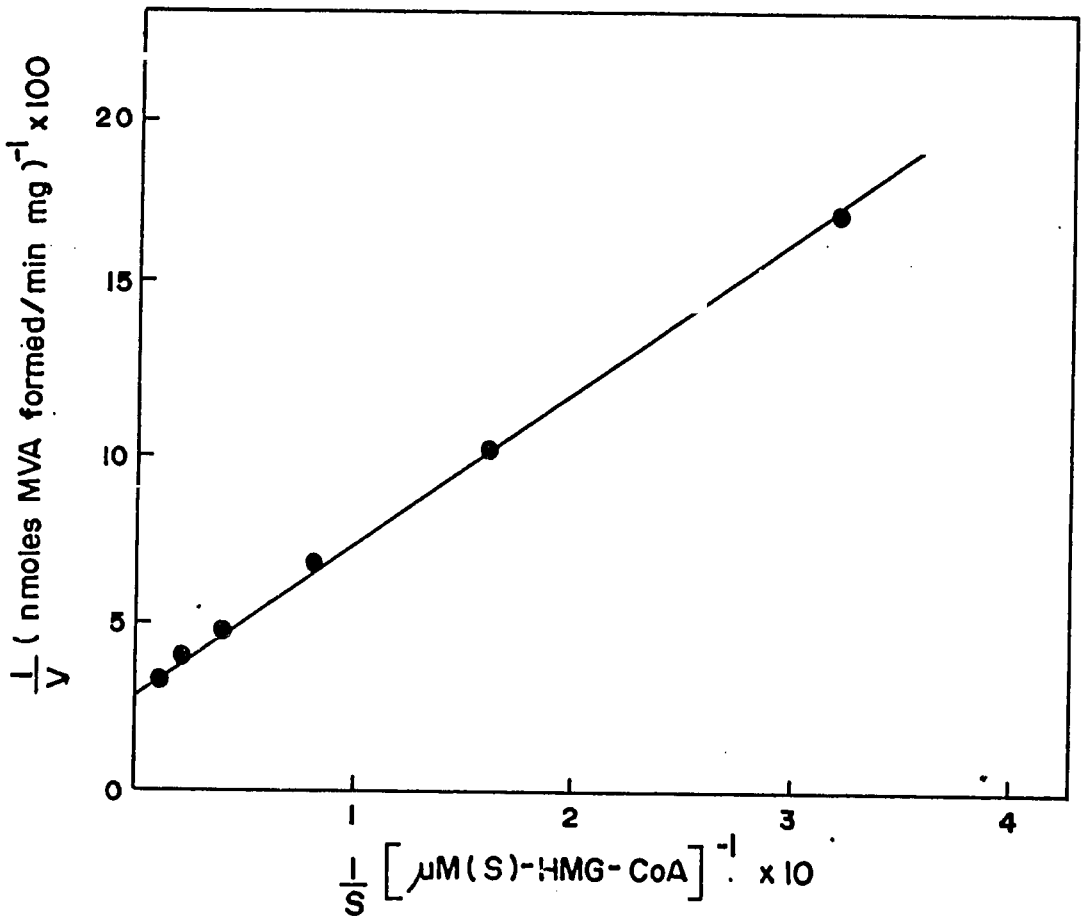

Fig.8 Affinity of purified HMGR for (S)-HMG-CoA. The assay mixture contains 10 ug of purified HMGR, 0.4 mM NADPH 0.1 M KCl, 10 mM DTT, 5 mM EDTA in K_xPO_4 buffer, pH 7.0

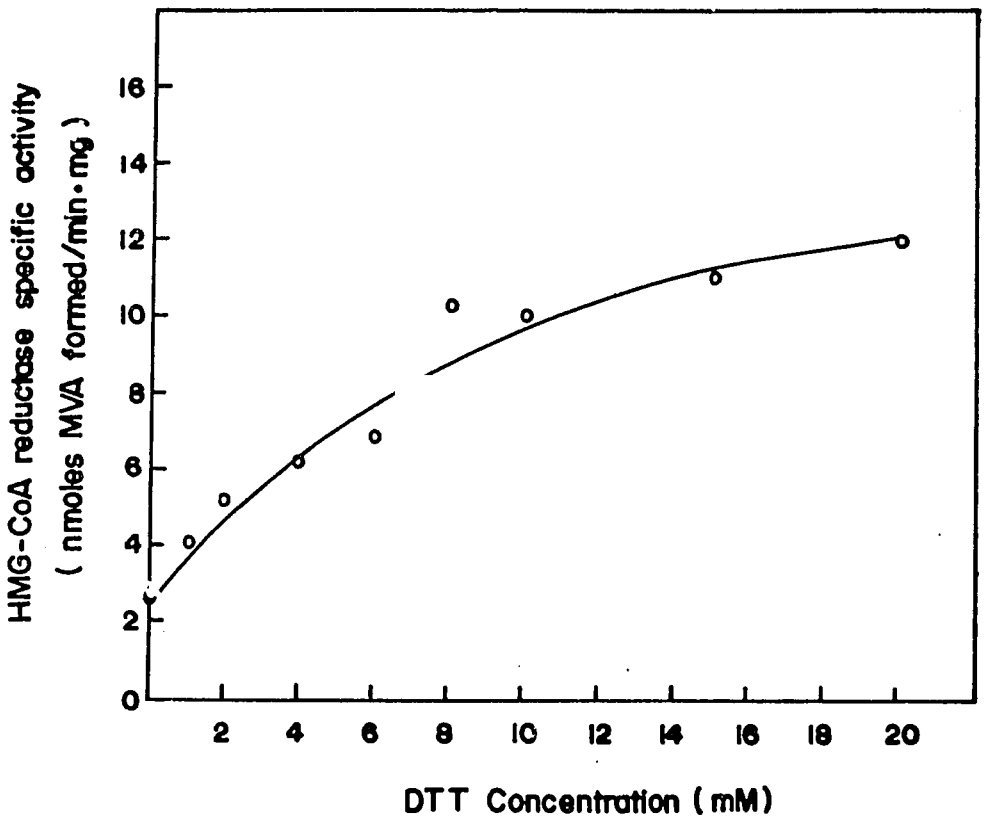

Fig.9 Effect of dithioltheitol on purified HMGR activity. The purified HMGR was suspended in buffer B (see methods) from which dithiothreitol was omitted and enzyme activity was then assayed.

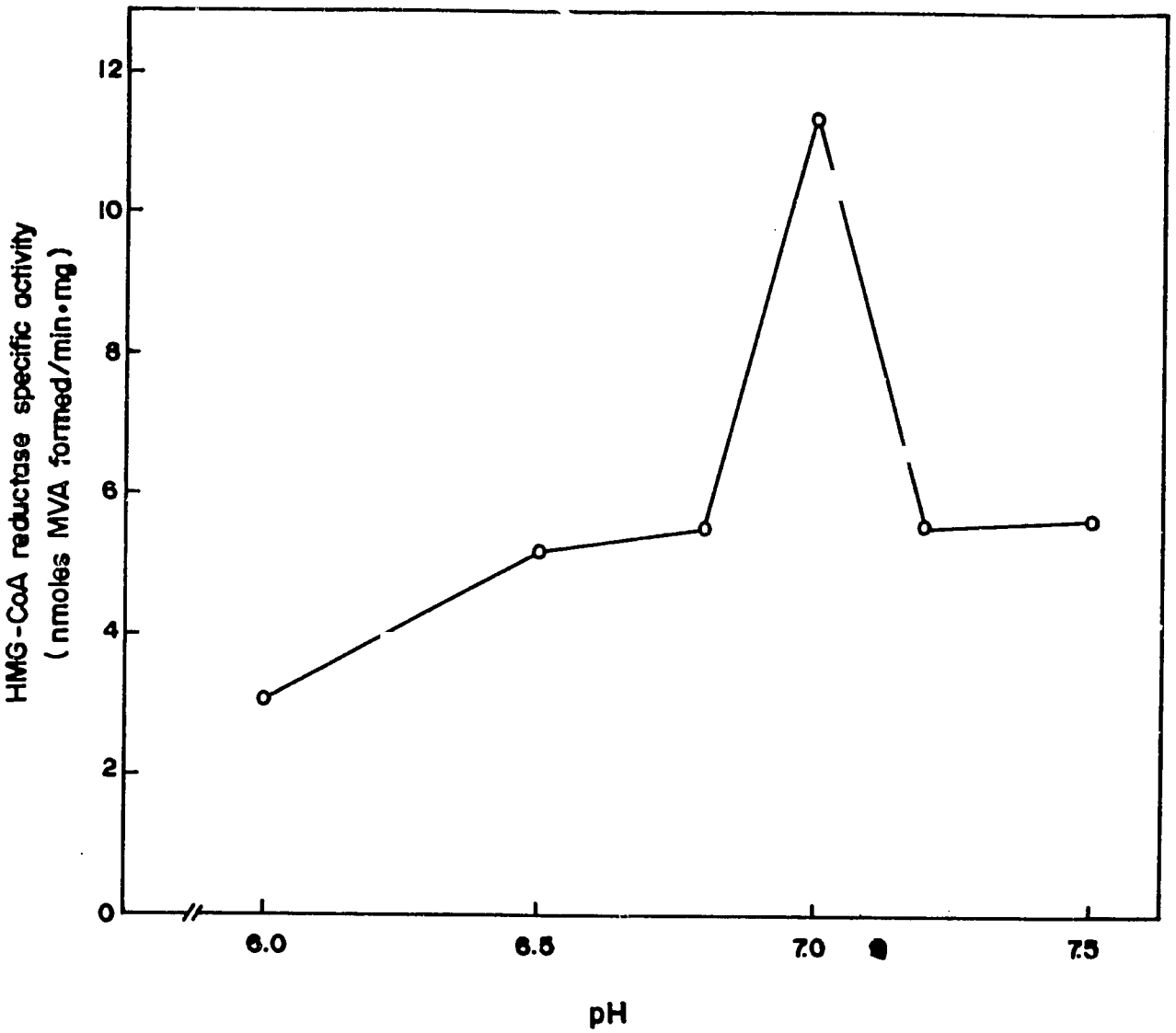

Fig.10 Activities of purified HMGR at different pHs. The enzyme was assayed in 0.1 M K_xPO_4 buffer (pH 7) containing 0.1 M KCl, 10 mM DTT 5 mM EDTA as described under methods.

Fig. 11a) Inner seed tegument slices as prepared from young immature *Hevea* seed.

Fig. 11b) Callus initiation from inner seed tegument at 2 weeks old.

Table 1
Purification protocol of *Hevea* HMG-CoA reductase.

Fraction/step	Total volume (ml)	Total protein (mg)	Total activity (nmoles MVA formed /min)	Specific activity (nmoles MVA formed /min.mg)	yield (%)	purification (fold)
Bottom fraction in Buffer B						
+ Brij w-1 (1%)	80	848	347.7	0.41	100	1
+ Glycerol (20%)	100	866.7	449.5	0.52	129.3	1.3
190,000 x g supernatant	95	570	404.7	0.71	116.4	1.78
0-40% $(NH_4)_2SO_4$	20.5	136.4	267.4	1.96	76.9	4.8
DEAE-Sephadex	109	24.8	227.4	9.19	65.4	22.4
0-90% $(NH_4)_2SO_4$	6	7.3	88.6	12.13	25.5	29.6
Affi-Gel Blue	10	1.5	47.3	31.95	13.6	77.9
HMG-CoA-hexane-agarose	4	0.37	39.1	105.65	11.3	257.7

Table II

Preliminary evaluation for rubber yield on *Hevea* germplasm

Sample no	Material	Clonal	Yield index*
1	RO/C/8	24/340	D
2	"----"	24/341	C
3	"----"	24/342	C^+
4	"----"	24/344	C
5	"----"	24/345	C^+
6	"----"	24/358	C
7	"----"	24/361	C
8	"----"	24/366	C^+
9	"----"	24/374	D
10	"----"	24/375	C
11	"----"	24/376	D
12	"----"	24/382	D
13	"----"	24/403	C^+
14	"----"	24/409	C
15	"----"	24/411	-
16	"----"	24/412	D
** 17	"----"	24/415	B^+
18	"----"	24/418	C+
19	"----"	24/419	D
20	"----"	24/423	D
21	"----"	24/425	D

Table II(continue)

Sample no.	Material source	clonal code	Yield index*
22	RO/C/8	24/435	C
23	"----"	24/437	D
24	"----"	24/439	C+
25	"----"	24/446	C
26	"----"	24/453	C+
27	"----"	24/454	C
**28	"----"	24/457	A+
29	"----"	24/458	C+
30	"----"	24/459	C
31	MT/IT/12	26/1	D
32	"----"	26/2	C+
33	"----"	26/9	C
34	"----"	26/12	D
35	"----"	26/14	C+
36	"----"	26/26	D
**37	"----"	26/32	A+
**38	"----"	26/34	A+
**39	"----"	26/37	B+
**40	"----"	26/49	B+
41	"----"	26/49	D
42	"----"	26/35	C
43	"----"	26/64	D

Table II(continue)

Sample no.	Material source	Clonal cod	Yield inde:
44	MT/TT/12	26/67	C
45	"----"	26/70	D
46	"----"	26/77	D
**47	"----"	26/79	B+
48	"----"	26/81	B
49	"----"	26/84	C
50	"----"	26/86	C+
51	"----"	26/118	D
52	"----"	26/122	C+
53	"----"	25/128	D
54	"----"	26/130	C
55	"----"	26/133	D

*Yield index was graded by adjusting from amount of latex being excreted from rubber leaflet upon cutting through leaf blade parallel to midrib vein. Grades A to D were given for high to low latex yield obtained.

**Selected for futher yield screening by HMGR assay.

Table III

Studies on latex HMGR activity from new *Hevea* germplasma.

Material sources	Clonal codes	HMGR activity per ml latex
Wickham	RRIM 600	6.71
RO/C/8	24/416	2.97
RO/C/8	24/457	4.83
MT/IT/12	26/32	4.63
MT/IT/12	26/34	7.50
MT/IT/12	26/37	3.71
MT/IT/12	26/45	4/51

Table IV

Effect of phytophthora toxin on callus growth

Clonal types	Toxin conc. (µg/ml)	Total cullus samples	Callus conditions	
			dead	alive
RRIM 600	0	6	1(16.7%)	5(83.3%)
	2.5	3	-(0%)	3(100%)
	25	3	-(0%)	3(100%)
	250	3	3(100%)	-(0%)
	275	2	2(100%)	-(0%)
	300	2	2(100%)	-(0%)
GT-1	0	6	-(0%)	6(100%)
	2.5	3	-(0%)	3(100%)
	25	3	-(0%)	3(100%)
	250	3	1(33.3%)	2(66.7%)

Table V

Effect of phytophthora toxin on callus initiation

Clonal types	Toxin conc. (µg/mg)	Total flower samples	Callus conditions after initiated		
			dead	alive	almost dead
RRIM 600	0	8	-	8	-
	125	4	3	-	1
	250	4	3	-	1
GT-1	0	8	-	8	-
	125	4	-	4	-
	250	4	-	4	-