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Characterization and Purification of a Microsomal 3-Hydroxy-3-Methylglutaryl-CoA Reductase in Rice Seedling

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Abstract : 3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR) catalyzes the conversion of HMG-CoA to mevalonic acid, the first intermediate of isoprenoid biosynthetic pathway in plants. The enzyme was solubilized with 0.4% Brij (polyoxyethylene ether) W-1 from a microsomal fraction of etiolated rice seedlings (*Oryza sativa* L.) in which its maximal activity was observed on the fourth day after germination. HMGR was purified to near homogeneity by employing $(NH_4)_2SO_4$ fractionation plus chromatographic procedures including DEAE-Sephadex A-50 and HMG-CoA-hexane-agarose affinity column. The size of the purified enzyme was estimated to be 55 kDa when judged by SDS-PAGE analysis with silver staining method. The apparent K_m and V_{max} values for HMG-CoA were determined to be 180 μM and 107 pmol/min/mg, and those for NADPH were 810 μM and 32.1 pmol/min/mg, respectively.(Received December 24, 1997; accepted January 15, 1998)

Introduction

3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR : EC1.1.1.34) catalyzes the major rate-limiting reaction of isoprenoid biosynthesis in animals. Because of its critical role in cholesterol biosynthesis, HMGR has been studied intensively in mammalian systems.[1,2] However, the amount and variety of end products dependent upon this pathway has been reported to be greater in plant than in animals.[3]

In plants, HMGR regulates the synthesis of some hormones such as abscisic acid and gibberelliin,[4] the phytol moiety of chlorophyll in plastid, phytoalexin and phytosterol in cytosol, and the side chain of ubiquinone in mitochondria.[5] Thus, it has been suggested that plant isoprenoid synthesis may be a highly compartmentalized process.[6] In addition, phytoalexins, phamacologically active agents and some plant isoprenoids such as fragrant oils and natural rubber are synthesized in this pathway.[7] The activity of HMGR has been identified mainly in dicotyledonous species such as pea, sweet potato, tobacco, radish, and *Hevea* rubber tree. Studies of monocotyledonous HMGR are limited in maize.[6,8] To investigate further on the regulation mechanism of the HMGR, it is necessary to purify this enzyme. However, the reports on the purification of HMGR in higher plants are limited only in three plants of pea seedling,[9] potato tuber,[10] and radish seedling,[11] this is because plant HMGRs are generally unstable and membrane-bound enzymes which make it to difficult to isolate. Especially, there has been no report on the purification of HMGR in monocotyledonous plants.

Cloning of several genes encoding plant HMGR has been reported in recent years. HMGR appears to be encoded by two genes in *Arabidopsis*,[12] three in *Hevea*,[13] and at least three in Potato.[14] The presence of multiple genes supports the hypothesis that the different isoforms of HMGR may be involved in subcellular distribution for isoprenoid biosynthesis.

The availability of HMGR from the plant sources may open more direct way of investigating the regulation of this enzyme. Furthermore, the HMGR can be applied as an interesting target enzyme for fungicides and the development of a new family of plant growth regulators. To investigate the regulation mechanisms of rice HMGR enzyme, we have studied on some properties of HMGR and the purification of the microsomal HMGR from rice seedlings.

Materials and Methods

Chemicals

The sources of the following isotopes and chemicals are

Key words : rice (*Oryza sativa* L.), 3-Hydroxy-3-Methylglutaryl-CoA reductase (HMGR), isoprenoid pathway
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indicated in parentheses: *RS*[-3-^{14}C]HMG-CoA (Amersham), [5-^{3}H]-mevalonic acid (MVA) lactone (NEN), DEAE-Sephadex A-50 and Hexane-agarose resin (Pharmacia), ethanolamine-HCl and diethyl ether (Merck), *RS*-HMG-CoA, MVA lactone, polyoxyethylene ether (Brij) W-1, polyvinylpyrophosphate (PVPP) and other chemicals (Sigma).

Preparation of rice microsomes

Rice (*Oryza sativa* L., cv. Ilpum) seeds were germinated and grown in a growth chamber at 37°C under the condition of complete darkness. Four-day-old etiolated seedlings were harvested with a scissors and quickly frozen in a liquid N_2.

Frozen seedlings were pulverized with precooled mortar and homogenized with a polytron homogenizer at maximum speed (2 strokes of 30s) in ice-cold buffer H [10 mM Tris (pH 7.0), 350 mM sucrose, 30 mM EDTA, 10 mM 2-mercaptoethanol, 0.1% BSA] containing 4% PVPP (w/v). The homogenates were squeezed through a layer of miracloth. The filterates were centrifuged at 12,000 g for 10 min at 4°C. The supernatant was centrifuged at 105,000 g for 90 min at 15°C. The precipitates (microsome) were suspended in buffer S1 [200 mM K_xPO_4 (pH 6.9), 25 mM DTT (added freshly)] and the glycerol was added to a final concentration of 20%. The suspension was stored at -80°C. The whole procedure was carried out at 4°C.

HMGR assay

The assay mixture consisted of 5 μ*l* 0.2 M DTT, 5 μ*l* 40 mM NADPH, and 400 μg microsomal protein in a final volume of 93 μ*l* and was preincubated for 5 min at 37°C. Substrate (1 μg/3 μ*l* *RS*-[3-^{14}C] HMG-CoA (0.06μCi) plus 44.6 μg/4 μ*l* *RS*-HMG-CoA) was added into the reaction mixture, and then incubated for 40 min in a shaking incubator at 37°C. The reaction was stopped by addition of 10 μ*l* 6 M HCl and 2 μ*l* 1 M MVA lactone. The sample was further incubated to lactonize for 15 min at room temperature. The reactants were centrifuged at 5,000 rpm for 5 min to remove membrane fragments. The 60 μ*l* supernatant was applied to Whatman LK 50F Silica gel TLC plate (20 cm × 20 cm). The plate was developed in diethylether: acetone (3:1, v/v) for 40 min. After iodine colorization, the region with Rf 0.4~0.5 was scraped and quantified by Beckman LS-200 liquid scintillation spectrometer. The activity was calculated as pmol of mevalonated formed per min and expressed as a relative activity.

Protein assay

The protein content of microsomes and solubilized enzyme preparations was determined by the modified Lowry method using trichloroacetic acid precipitation (Sigma).

Solubilization of Microsomal HMGR

The microsomal suspension that had been stored at -80°C was thawed at room temperature and incubated at 37°C for 20 min. Buffer S2 [100 mM K_xPO_4 (pH 6.9), 5 mM EDTA, 100 mM KCl, 10 mM DTT (added freshly)] and 6% Brij W-1 were added to this slurry (final concentration of 0.4%) and the samples were resuspended with a teflon homogenizer. The suspension was incubated for 30 min at 37°C, then glycerol was added (to a final concentration of 20%) and further incubated for 30 min at 37°C followed by a centrifugation at 105,000 g for 60 min at 15°C. In this solubilized fraction, ammonium sulfate was slowly added to a final concentration of 40%. The mixture was kept at 4°C for 40 min, then centrifuged at 25,000 g for 20 min at 4°C. The precipitates were redissolved in buffer R [50 mM K_xPO_4 (pH 6.9), 5 mM EDTA, 0.1% Brij W-1, 10 mM DTT (added freshly)] and dialyzed with the same buffer at 4°C. The resulting yellow solution was used for the further purification of the microsomal HMGR.

Purification of HMGR

The solubilized and dialyzed microsomal fraction was applied to a DEAE-Sephadex chromatography (3 cm × 30 cm) which was equilibrated with buffer R. After washing the column with the same buffer, proteins were eluted with the same buffer containing a linear gradient of 1 M KCl. The elution volume of each fraction was 10 m*l*. The active fractions from this chromatography was pooled and dialyzed with buffer R.

Affinity column was prepared with 1 g hexane-agarose resin and 12.5 mg HMG-CoA containing 0.2μCi of *RS*-[3-^{14}C]HMG-CoA. The coupling efficiency of the affinity resin was 48 to 60%. The pooled active fractions obtained from DEAE-Sephadex A-50 chromatography was concentrated with amicon concentrator (model 8050). The active samples were applied onto the affinity column that had been equilibrated with buffer R. This step was repeated twice. The proteins bound to the gel were eluted with the step gradient buffer R; buffer R1 (100 mM KCl in R), buffer R2 (100 mM KCl plus 5 mM NADPH in R), buffer R3 (500 mM KCl in R), and buffer R4 (1000 mM KCl in R).

Results and Discussion

Subcellular distribution and developmental expression of HMGR in etiolated rice seedling

The subcellular localization of HMGR has important regulatory implications for the potential involvement of this enzyme in independent subcellular pathways.[15-17] Reports on the presence of HMGR in several cell compartments (endoplasmic reticulum, mitochondria, and plastid) supported that the HMGR activity of distinct cell fractions has different kinetic parameters and exhibit differential regulation.[18] To localize the enzyme activity in etiolated rice seedlings, subcellular fractionations were performed by the stepwise centrifugations. Of the enzymes present in mitochondria, microsome and cytosols, the microsomal fraction showed the highest HMGR activity. The enzymatic activity of mitochondrial and cytosolic HMGR were 30% and 17% that of the microsomal HMGR, respectively (Fig. 1). This result supports the proposal that separate pathways for isoprenoid biosynthesis may exist in rice.

To examine the developmental expression of the HMGR, the microsomes were prepared at different time point (*e. g.* one to nine days after germination) from etiolated seedlings and analyzed for their activities. The activity of HMGR reached the peak on fourth day after germination, then decreased slowly (Fig. 2). Since the fourth day after germination seemed to be a time of activated cell division, increase of HMGR activity in rapidly dividing cells suggests that mevalonate derivatives play a very important role in cell division and sterol biosynthesis required formation of cell membrane is promoted by HMGR expression.[19] Inhibition of HMGR by mevinolin (specific inhibitor of HMGR) also appears to block cell division in plant tissues.[20]

Enzymatic characteristics of rice microsomal HMGR

To determine the properties of the microsomal enzyme with respect to kinetic parameters and cofactor requirement, series of enzyme assay were carried out under various physiological conditions. The optimal temperature and pH of the microsomal HMGR were 37°C and pH 6.9 (data not shown). The apparent K_m values of the crude microsomal HMGR for the HMG-CoA and NADPH from Lineweaver-Burk plots were found to be 180 μM and 810 μM, respectively. The comparision of the apparent K_m value for the HMG-CoA and NADPH of rice, potato tuber,[10] and radish seedling[11] are shown in Table 1. The K_m value of the crude microsomal HMGR from rice seedlings is significantly higher than those of membrane-bound HMGR from potato tuber, and purified HMGR from radish seedling.

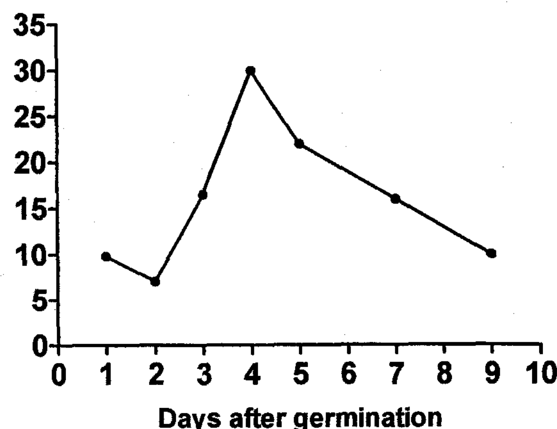

Fig. 2. Change of microsomal HMGR activities during rice seedling germination. Each microsomal fraction was prepared from etiolated rice seedling.

HMGRs found from the animal and the pea seedling have been known to be NADPH-dependent enzyme.[21] To examine whether the rice HMGR uses NADPH as a proton donor, enzyme assay was carried out in the presence of either NADPH or NADH. As shown in Fig. 3, the microsomal HMGR from rice seedlings was found to be NADPH-dependent enzyme.

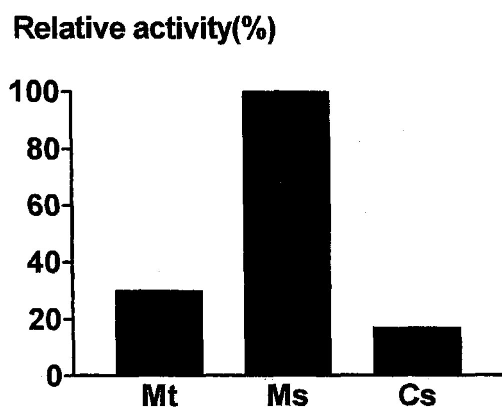

Fig. 1. Subcellular distribution of HMGRs in rice seedling. Each fraction was prepared from rice seedling by differential centrifugation. Mt : Mitochondria, Ms : Microsome, Cs : Cytosol

Solubilization and purification of rice microsomal HMGR

To solubilize the microsomal HMGR, Brij W-1 and Tween 40 that had been used extensively in plant were em-

Table 1. The comparision of Michaelis-Menten constants of HMGRs from rice seedling, potato tuber, and radish seedling.

Substrate	Rice seedling		Potato tuber[a]	Radish seedling[b]
	K_m	V_{max}	K_m	K_m
HMG-CoA	180 μM	107 pmol/min/mg	38 μM	1.5 μM
NADPH	810 μM	32pmol/min/mg	89 μM	27 μM

[a] Kondo and Oba, 1986
[b] Bach *et al.*, 1986

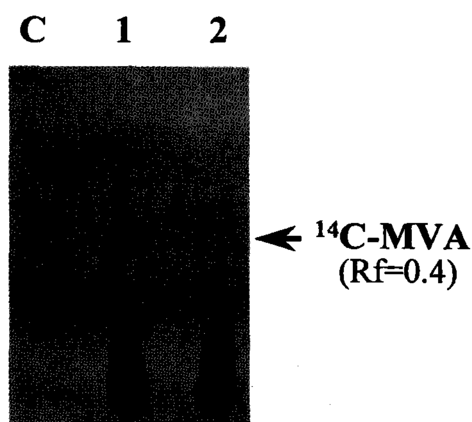

Fig. 3. Autoradiograph demonstrating the NADPH-dependent HMGR reaction. The rice microsomal HMGR was assayed in the presence of either NADPH (Lane 1) or NADH (Lane 2) as a cofactor. C : Control (^{3}H-MVA)

ployed in this experiment. Brij W-1 (0.4%, w/v) was found to solubilize the microsomal HMGR to 95% and also to enhance the HMGR activity, while Tween 40 was found to be less efficient (data not shown). Similar observation has been reported on the solubilization of the radish membrane-bound HMGR with Brij W-1.[11] Solubilized HMGR activity was stable in buffer S2 containing 10 mM DTT and 20% glycerol. The solubilized protein (223 mg) was precipitated with 40% ammonium sulfate. The precipitated fraction from this procedure resulted in about 4-fold purification over the crude microsomal suspension (Table 2). The ammonium precipitated fractions were further purified through the chromatographic procedures by employing DEAE-Sephadex A-50 (Fig. 4) and HMG-CoA-hexane-agarose affinity column (Fig. 5). 40% concentration of ammonium sulfate resulted in some loss of HMGR activity but also in removal of an interfering enzyme activity.

Table 2. Purification of the microsomal HMGR from rice etiolated seedling.

Fraction/ step	Total volume (ml)	Total protein (mg)	Total activity (pmol/ min)	Specific activity (pmol/min /mg)	Purification fold	Yield (%)
Microsomal suspension	19.8	425.9	17,015	40	1.0	100.0
Solubilized supernatant + Brij W-1 + 105,000xg	23	222.9	7,105	31.9	0.8	41.8
0-40% $(NH_4)_2SO_4$	6.5	33.9	4,294	126.6	3.2	25.2
DEAE-Sephadex A-50	8.0	3.2	4,121	1,278.1	31.0	24.2
HMG-CoA-hexane-agarose	5.0	0.05	1,216	24,312.5	609	7.1

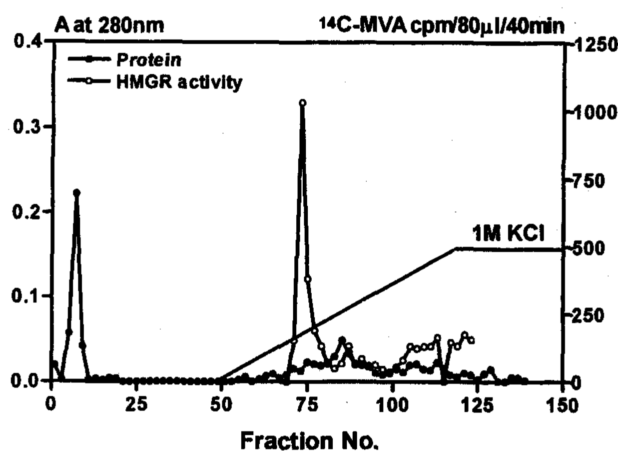

Fig. 4. Elution profile of the HMGR from DEAE-Sephadex A-50 chromatography. Solubilized microsomal fractions were loaded onto a DEAE-Sephadex A-50 column that had been equilibrated with buffer R. described in the materials and methods. Proteins were eluted with the same buffer containing 1 M KCl.

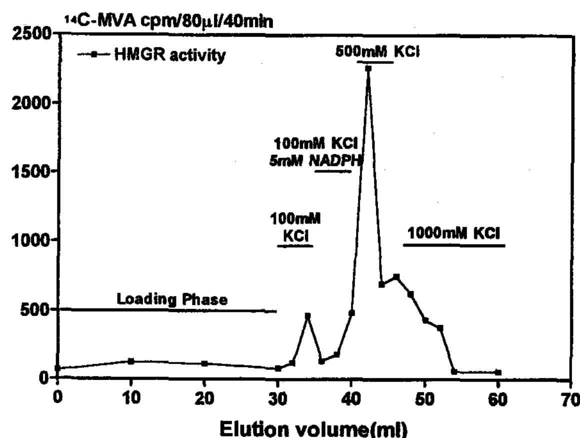

Fig. 5. Elution profile of the HMGR from the affinity chromatography. Active fractions from DEAE-Sephadex A-50 chromatography were loaded onto a HMG-CoA-hexane-agarose affinity column which had been equilibrated with buffer R. Proteins were eluted with the same buffer containing four different concentrations of KCl; 100 mM KCl, 100 mM KCl plus 5 mM NADPH, 500 mM KCl, and 1000 mM KCl. The relative absorbance at 280 nm was too low to be measured.

The specific activity of the purified enzyme from the final round of the affinity column was about 25,000 pmol mevalonate formed/min/mg protein and the overall recovery from microsomal fraction was about 7.1% (Table 2).

Fig. 6 shows that molecular weight of the purified HMGR subunit was determined to be 55 kDa by SDS-PAGE analysis. This size of HMGR is quite similar to that of potato tuber HMGR subunit.[10] The active form of potato tuber HMGR has a dimeric structure of 110 kDa composed of two identical subunits of approximately 55 kDa.[10] We were not able to define a molecular weight of the native form of rice microsomal HMGR.

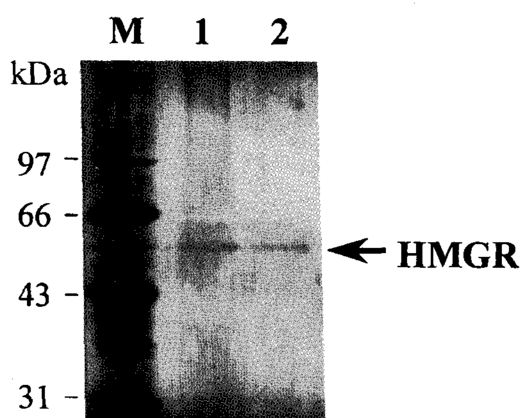

Fig. 6. Determination of the molecular weight of the purified HMGR from rice seedlings. M : Low molecular weight size marker, Lane 1, 2 : the active fractions from the affinity chromatography. Electrophoresis was carried out on 10% SDS-PAGE which was subject to silver staining.

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벼 HMG-CoA 환원효소의 특성연구

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초 록 : 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) 환원효소는 식물의 병원균 방어물질 (phytoalexin), 광합성 색소 (the phytol of chlorophyll), 성장 호르몬 (abscisic acid와 gibberellin 등) 및 스테롤 (phytosterol) 등의 생합성에 관여하는 주효소이다. 암조건 하에서 발아 후 4일째의 벼 유묘 microsome을 재료로 비이온성 detergent인 Brij W-1 (final 0.4%)을 사용하여 가용화 시킨 후, DEAE-Sephadex A-50 크로마토그래피 칼럼과 hexane-agarose를 matrix로 기질인 HMG-CoA를 결합시켜 제조한 친화성 크로마토그래피 칼럼을 이용하여 세포막 결합효소인 HMG-CoA 환원효소를 정제하였다. 정제된 HMG-CoA 환원효소의 최종 회수율은 7.14% 였고, 분자량은 10% SDS-PAGE에서 55 kDa이였다. HMG-CoA 환원효소의 최적 반응 온도는 37℃, 최적 반응 pH는 6.9였고, 기질인 HMG- CoA에 대한 HMG-CoA 환원효소의 K_m 및 V_{max} 값은 *180* μM과 107 pmol/mg, 수소공여체인 NADPH에 대한 K_m과 V_{max}값은 810 μM과 32.1 pmol/min/mg이였다.

찾는말 : 벼, HMG-CoA 환원효소

*연락저자