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Expression of catalytically active radish 3-hydroxy-3-methylglutaryl coenzyme A reductase in *Escherichia coli*

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Two fragments of a cDNA encoding radish 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) were cloned into the vector pET-8c and expressed in *Escherichia coli*. The large fragment, encoding both the membrane and the cytosolic domains, was expressed at low level, essentially as an insoluble protein without enzymatic activity. In contrast, the fragment encoding only the cytosolic domain was expressed at a high level in a catalytically active form. The amount of soluble active enzyme in cell-free extracts of *E. coli* dramatically increased when the temperature during the induction was lowered from 37°C to 22°C.

HMG-CoA reductase; Isoprenoid biosynthesis; Cloned enzyme expression; *Rhaphanus sativus*; *Escherichia coli*

1. INTRODUCTION

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR; EC 1.1.1.34) catalyzes the synthesis of mevalonate from 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), considered to be a major rate-limiting step in plant isoprenoid biosynthesis [1,2]. The main subcellular location of plant HMGR appears to be the membrane of the endoplasmic reticulum, although enzyme activity has also been reported to be associated with mitochondrial and plastid membranes [3]. Recently, cDNA clones corresponding to plant HMGR have been obtained from *A. thaliana* [4,5], radish [6], tomato [7] and *H. brasiliensis* [8]. Based on the data available from the enzymes presently cloned and sequenced [4–6], 3 different structural regions have been defined in the protein: the N-terminal or membrane domain (containing two putative transmembrane spanning regions), the linker region, and the C-terminal or cytosolic domain (bearing the catalytic site) [4–6,9]. Hitherto, a detailed functional and structural in vitro-analysis of the enzyme has been mainly hampered by difficulties in obtaining large amounts of pure enzyme from plant sources [10]. In addition, the presence of different HMGR isozymes that are known to occur in plants [9] certainly makes their individual characterization difficult by using conventional protein purification techniques. This can now be overcome by producing large amounts of the desired enzyme through the expression of cDNA clones in a heterologous system. Here we report the expression of high levels of catalytically active radish HMGR in *E. coli*.

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2. MATERIALS AND METHODS

2.1. *Materials*

Restriction enzymes, Klenow polymerase and bacteriophage T4 DNA ligase were obtained from Boehringer-Mannheim. The bacterial expression vector pET-8c and *E. coli* strains HMS174 and BL21(DE3) were kindly provided by F.W. Studier (Brookhaven National Laboratory, Upton, NY). [^{35}S]Methionine and [methyl-^{3}H]HMG-CoA were from New England Nuclear.

2.2. *Construction of the expression vectors pHMGR63 and pHMGR45*

Plasmid pcRS3.1 contains a cDNA for radish HMGR comprising a 1749 base pair (bp) open reading frame flanked by 45 bp at the 5′-end and 230 bp at the 3′-end [6]. From this cDNA clone we made two constructions in the expression vector pET8c [11] (Fig. 1). DNA from pcRS3.1 was digested with *Eco*RV, and the resulting fragment was ligated into *Nco*I-restricted and Klenow polymerase-treated pET-8c. The resulting expression plasmid was designated pHMGR63. The same *Eco*RV fragment was further digested with *Bam*HI, and the resulting *Bam*HI-*Eco*RV fragment was filled in by Klenow polymerase treatment, and ligated into *Nco*I-restricted and Klenow polymerase-treated pET-8c. The resulting expression plasmid was designated pHMGR45. The clones of interest were initially selected in *E. coli* HMS174 and further introduced in *E. coli* BL21(DE3) for expression studies.

2.3. *Expression in* E. coli

E. coli BL21(DE3) harboring pHMGR63, pHMGR45 or pET-8c were grown at 37°C on M9ZB medium with 100 μg/ml ampicillin to A_{600} = 0.6, and then induced by the addition of 0.4 mM isopropyl-

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β-D-thiogalactoside (IPTG), as described [11]. Rifampicin (200 μg/ml of culture) was added 30 min after induction, and cells were incubated at the indicated temperatures for different time intervals. After induction, cells from 1 ml of culture were collected by centrifugation (7000 × g, 5 min, 4°C), resuspended in 400 μl of 100 mM sucrose, 40 mM potassium phosphate, pH 7.2, 10 mM EDTA, 50 mM potassium chloride, 10 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 0.2% (v/v) Triton X-100, and disrupted (1 min/ml of suspension) in an ultrasonic disintegrator (MSE, 60 W) while being chilled in a −10°C bath. Cell debris was removed by centrifugation (15000 × g, 20 min, 4°C) and the supernatant was used either for enzyme assays or protein fractionation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. *Radiolabeling of proteins*

The synthesis of induced proteins was monitored by pulse-labeling with [^{35}S]methionine as follows: after the addition of rifampicin to the IPTG-induced cultures, [^{35}S]methionine was added to a final concentration of 20 μCi/ml. At different times, cells from aliquots of 1 ml were harvested (7000 × g, 5 min, 4°C), and resuspended in 100 μl of 4% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.02% (w/v) bromophenol blue in 60 mM Tris-HCl, pH 6.8. After 3 min at 100°C, aliquots of 20 μl were subjected to SDS-PAGE. For the analysis of soluble radiolabeled proteins, cell-free extracts were prepared by sonication, as described above, and 50 μl aliquots were subjected to electrophoresis using the same sample buffer solution.

2.5. *Assay of enzyme activity*

HMGR activity was determined by the radiometric assay described by Mayer et al. [12]. One unit of HMGR activity is defined as the amount of enzyme which converts 1 nmol of HMG-CoA into mevalonate in 1 min at 37°C.

2.6. *SDS-PAGE and detection of proteins in gels*

Denaturing polyacrylamide gel electrophoresis was performed by the procedure of Laemmli [13], using a 10% polyacrylamide running gel and a 3.5% stacking gel. After electrophoresis, proteins were detected: (i) by Coomassie blue staining, (ii) by fluorography [14], and (iii) by Western blot [15] using anti-rat liver HMGR-specific antibodies kindly provided by F.G. Hegardt (Unitat de Bioquímica, Facultat de Farmacia) and ^{125}I-protein A.

2.7. *Determination of protein*

Protein concentration was determined by the method of Lowry et al. [16] with bovine serum albumin as a standard.

3. RESULTS AND DISCUSSION

In the present paper we report the expression of large amounts of the enzymatically active radish HMGR. Two fragments of a radish HMGR cDNA were cloned into vector pET-8c (Fig. 1) and expressed in *E. coli*. pHMGR63 contained a fragment coding for a form of HMGR comprising both the membrane and the cytosolic domains (residues 3–583) (HMGR63; deduced M_r = 62720). The fragment cloned into pHMGR45 codes for amino acids 160–583, which constitutes a truncated form of HMGR comprising only the cytosolic domain (HMGR45; deduced M_r 44806). In both cases, the cDNA fragments were directly attached to the translation initiation codon of pET-8c, and placed under the control of the φ10 bacteriophage promoter.

After 4 h of induction at 37°C in the presence of [^{35}S]methionine, cells harboring pHMGR45 or pHMGR63 synthesized two new proteins migrating at an apparent M_r of 47 kDa and 63 kDa, respectively (Fig. 2), in close agreement with the predicted values. These proteins were not present after the induction of cells harboring control pET-8c (Fig. 2). It should be pointed out that HMGR45, which represents the major cell protein, was more efficiently expressed than HMGR63, as shown after SDS-PAGE and Coomassie blue staining (Fig. 2). To further confirm their identity, these proteins were subjected to a Western blot analysis using antibodies raised against rat liver HMGR. Both proteins were recognized by these antibodies (Fig. 2). This is in contrast to the lack of immunoinhibition by anti-yeast HMGR and anti-rat liver HMGR IgG of detergent-solubilized HMGR from radish seedlings [10]. The reason for this latter observation could have been the blockage of antigenic determinants of the protein by the detergent. Close inspection of the induced proteins of both pHMGR45 and pHMGR63 revealed in each case two additional induced polypeptides (M_r = 31177 and M_r = 32823), that were immunochemically related to HMGR protein, as deduced from the Western blot analysis (Fig. 2). Presumably, these polypeptides represent two subfragments of HMGR initiated at the internal ATG codons in positions 287 and 272, which are casually preceded by sequences that could act as ribosome binding sites [17].

When the induction was performed at 37°C, HMGR activity (2.3 U/mg) was only detected in the soluble fraction of extracts from cells harboring pHMGR45 (Fig. 3A). This enzyme activity was completely blocked by mevinolin, a highly specific inhibitor of HMGR [18,19], thus demonstrating that in our assay system we were exclusively measuring HMGR activity (data not shown). HMGR63 was largely localized in the insoluble protein fraction, as well as most of HMGR45. This latter observation was unexpected, especially in the case of HMGR45, since it is well documented that the cytosolic domain of HMGR behaves as a soluble protein. In fact, HMGR purified from different sources corresponds to a soluble proteolytic fragment comprising the cytosolic domain [10,12,20,21].

It is known that high levels of expression of foreign proteins in *E. coli* frequently result in the formation of large amounts of insoluble protein as compared to the soluble product [22]. As an approach to overcoming the insolubility of HMGR45 and HMGR63, we decreased the temperature of induction, because it has been described that the temperature-dependent reduction of the rate of protein synthesis usually results in an enhanced yield of the soluble expressed proteins [22].

When cells harboring pHMGR63 were induced at 22°C and harvested at different time intervals, low, but measurable, levels (<0.05 U/mg) of HMGR activity were detected. Even when the induction temperature

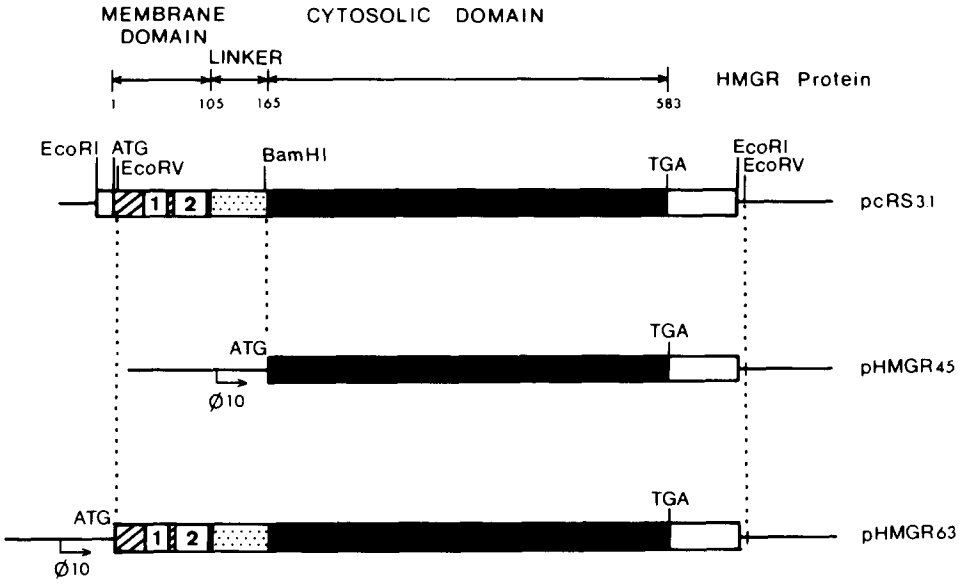

Fig. 1. Construction of the plasmid vectors pHMGR45 and pHMGR63 for expression of radish HMGR. Boxes delineating the location of the membrane domain (hatched), including the two putative transmembrane spanning regions (1 and 2), the linker region (stippled), the catalytic domain (solid) and the 5′ and 3′ non-coding regions (open) are shown. The coding region of HMGR is also shown at the top of the figure, and numbers show positions of the amino acids defining the different domains of the protein. Position and orientation are shown for the T7 RNA polymerase promoter (ϕ10).

was reduced to 22°C, most of HMGR63 protein remained associated with the insoluble fraction. Nevertheless, we hope that HMGR63 can be recovered in an enzymatically active form after its solubilization and/or reconstitution into its active conformation, which is under current study. Although there exist several factors that account for the insolubility of foreign proteins overexpressed in *E. coli* [22], in this

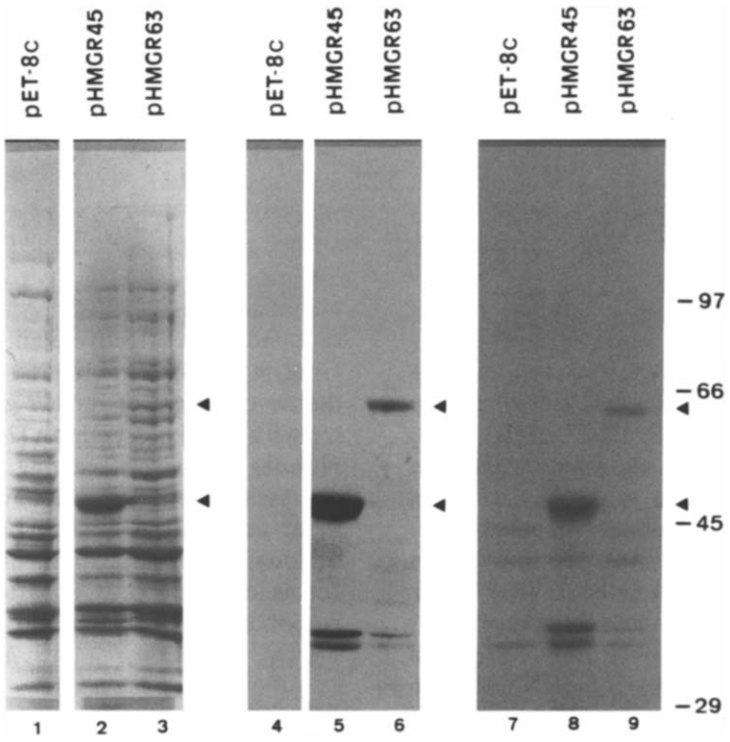

Fig. 2. Analysis of total protein (approx. 75 μg/lane) from transformed *E. coli* cells after 4 h of IPTG induction at 37°C. The cells were harboring the plasmids indicated at the top of each lane. Proteins were identified by staining with Coomassie blue (1–3), fluorography (4–6) or immunoblotting (7–9). Arrowheads show the position of the new proteins produced. Molecular masses of standards are indicated in kDa.

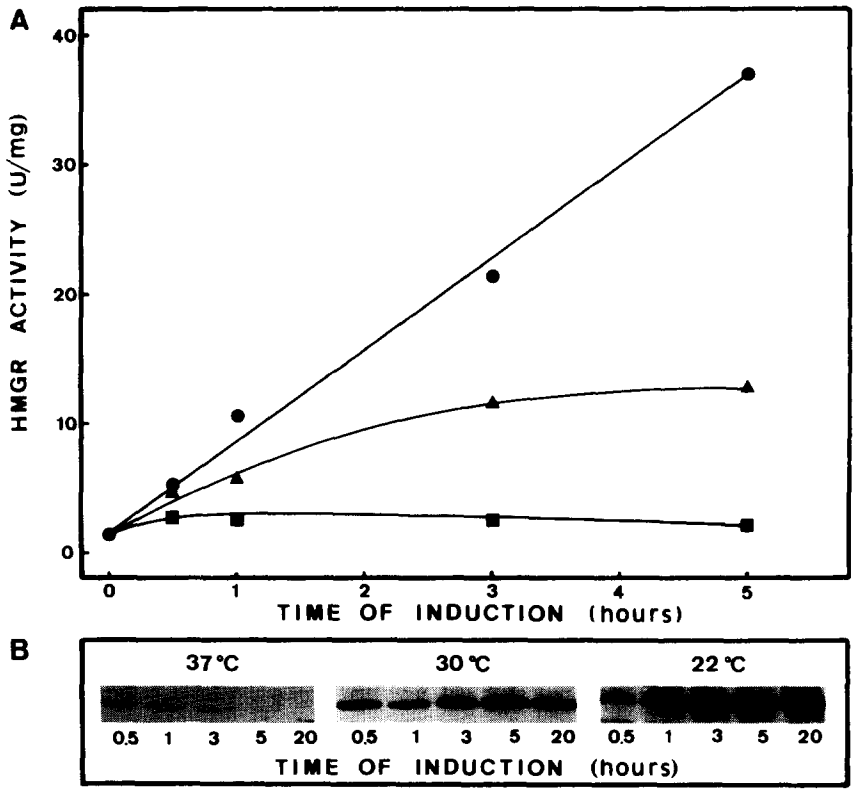

Fig. 3. (A) Effect of the induction temperature on the level of HMGR activity. HMGR activity was determined in the soluble fraction of extracts from *E. coli* cells harboring pHMGR45 at different times after IPTG induction and at the following temperatures: 22°C (●), 30°C (▲) and 37°C (■). (B) Effect of the induction temperature on the accumulation of soluble HMGR45. The soluble fraction of extracts from *E. coli* cells harboring pHMGR45 was obtained at different time intervals after induction by IPTG, in the presence of [^{35}S]methionine. The samples were analyzed by SDS-PAGE followed by fluorography. Only the part of the fluorogram is shown which contains the HMGR45 band.

specific case the behavior of HMGR63 could presumably be attributed to the presence of the two highly hydrophobic sequences within the membrane domain [6,9].

In the case of cells harboring pHMGR45, HMGR activity increased when the induction was performed at temperatures below 37°C (Fig. 3A). At 30°C HMGR activity reached a maximum after 5 h of induction (12.5 U/mg) (Fig. 3A), and remained constant for up to 20 h after induction (not shown). Interestingly, at 22°C the activity increased in a linear manner during the first 5 h of induction (Fig. 3A) and it continued to increase (although not in a linear manner) for up to 20 h (not shown). It is noteworthy that the high value of HMGR specific activity (93 U/mg) found in cell-free extracts after 20 h of induction at 22°C, is several orders of magnitude higher than those reported in enzyme extracts from various eukaryotic sources [3,10,21]. No HMGR activity was detected after induction of cells harboring pET-8c under all the conditions tested. The higher levels of HMGR activity detected in the soluble fraction of cells harboring pHMGR45 induced at 22°C or 30°C clearly correlated with the increase of soluble HMGR45 detected in this fraction. Fig. 3B shows the time course of the synthesis of soluble HMGR45 in cells induced at 22, 30 and 37°C.

The high efficiency of the expression system used, together with the high stability of HMGR45 within *E. coli* cells, makes it possible to easily obtain large amounts of the catalytically active cytosolic domain of plant HMGR. Here we present a virtually unlimited source of HMGR protein, from which either the truncated or full-size enzyme could be purified, thereby allowing for further detailed functional and structural analysis, including in vitro-NMR and site-directed mutagenesis. Since mevinolin, a fungal metabolite, was revealed as an effective plant-growth inhibitor [23,24], HMGR has been considered as a promising target for the development of further growth regulators interfering with sterol biosynthesis [24,25]. In the search for inhibitors exclusively reacting with plant HMGR(s) our expression system might provide the basis for a convenient test system. It also paves the way for the individual characterization of the different isozymic forms of plant HMGR, after expression of the corresponding cDNA clones in bacterial cells.

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