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Enhanced Production of Coenzyme Q10 by Overexpressing HMG-CoA Reductase and Induction with Arachidonic Acid in *Schizosaccharomyces pombe*

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Abstract Coenzyme Q10 (CoQ10) is a vitamin-like substance which plays a crucial role in the respiratory chain ranging from bacteria to humans and in the radical scavenging in human body. In this study, the full-length *hmgR* gene (encoding 3-hydroxy-3-methyl-glutaryl-CoA reductase, HMG-CoA reductase) was cloned and overexpressed in *Schizosaccharomyces pombe*. Using the pREPG yeast depressed under the thiamine as the control, CoQ10 contents increased up to 2.68 and 3.09 times when recombinant cells were incubated without and with arachidonic acid, respectively. It demonstrated that arachidonic acid could upregulate the activity of HMG-CoA reductase and that *hmgR* gene played a significant role in CoQ10 biosynthesis. So, it has an importance to be utilized for fermentation.

Keywords HMG-CoA Reductase · *Schizosaccharomyces pombe* (fission yeast) · Coenzyme Q10 · Arachidonic acid

Introduction

Coenzyme Q10 (CoQ10), also known as ubiquinone, is a vitamin-like substance that is naturally synthesized in the body. It plays an important role in the electron transport system from bacteria to humans and in the antioxidant property [1]. Recently, it has been widely used in pharmaceuticals, cosmetics, and foods due to its various physiological activities [2–4]. It can be mainly obtained by three methods: extraction from animal tissues, chemical synthesis, and microorganism fermentation [5]. Biosynthesis route is considered as a promising method because of low costs and easy production process. But, it is still limited due to the low productivity of industrial strains. So, it is essential to analyze the key metabolic pathway of CoQ10 to improve its yield. *Schizosaccharomyces pombe* (fission yeast) is a popular model system for producing a variety of proteins, and it is also expected

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to be a model to study CoQ10 biosynthesis. CoQ10 molecule is comprised of a benzoquinone ring and ten isoprene units side chain. CoQ10, carotenoid, and sterols share the same precursor of isoprene. Therefore, production of isoprene unit is presumed to be the rate-limiting step (Fig. 1).

The 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase) catalyzes an irreversible conversion from 3-hydroxy-3-methyl-glutaryl-CoA to mevalonate and ultimately to isopentenyl diphosphate (IPP), which serves as a precursor for CoQ10 side chain biosynthesis. It was reported that mevalonate pathway was the only route to obtain the isoprene in vivo in human [6] and yeast [1]. Therefore, HMG-CoA reductase was postulated as a key rate-limiting enzyme in mevalonate pathway, which had been studied by many research groups [7]. To date, *S. pombe* is the only unicellular eukaryote that has been found to contain a single HMG-CoA reductase gene [8]. Consequently, *S. pombe* may provide important opportunities to study different aspects of this enzyme to regulate the isoprends end-products biosynthesis. In the animal cell, the catalyzed reaction of HMG-CoA reductase was irreversible so it was assumed that there was obvious relevance between HMG-CoA reductase activity and synthesis of CoQ10, one of IPP end-products.

It was reported that inhibitors of HMG-CoA reductase, such as statins, were used as efficient drugs to cure high cholesterol disease [9]. Statins inhibited metabolic flux of mevalonate pathway to decrease the accumulation of cholesterol. Therefore, it was considered as a key step in cholesterol biosynthesis. In addition, Shimada used strong promoter to overexpress HMG-CoA reductase in *Candida utilis* to gain high yield of carotenoid [10]. Carotenoid has a similar biosynthesis pathway with CoQ10, but there was

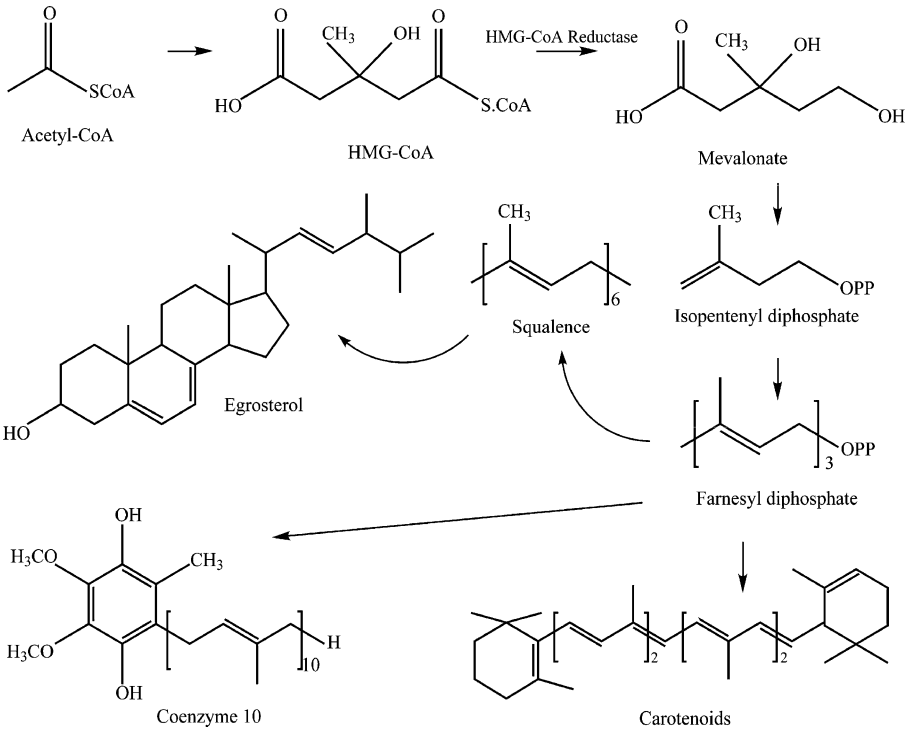

Fig. 1 Ubiquinone biosynthesis pathway in eukaryotic microorganism

no report about the relation between overexpressing HMG-CoA reductase and CoQ10 yield.

Manuel [11] found that the transcriptional level of one of HMG-CoA reductase gene family in plant increased with the injection of arachidonic acid. In addition, a yeast strain was very sensitive to polyunsaturated acids because of the deficiency of CoQ10, and the strain could die with the treatment with polyunsaturated acids [12]. However, there were no reports on CoQ10-overproduced yeast with the treatment of arachidonic acid. In the present study, *HmgR* gene was overexpressed and CoQ10-overproduced recombinant yeast was obtained. The effect of arachidonic acid on HMG-CoA reductase is investigated.

Materials and Methods

Materials

Restriction enzymes, LA Taq DNA polymerase and DNase I (RNase Free), were purchased from Takara (Japan); T4-DNA Ligase and reverse transcriptase from Promega (USA); Plasmid extraction Kit from Omega (USA); and Genomic DNA extraction Kit from Tiangen (China). Arachidonic acid and all other chemicals of analytical grade were from Beijing Chemical Regents Company. Leucine and thiamine were purchased from Sigma (USA).

Strains, Plasmids, and Culture Conditions

Escherichia coli DH10B was kindly provided by Dr. Xia (Chinese Academy of Sciences, Beijing, China). It was used as a host for plasmid construction and had superiority to contain a bigger shuttle vector. PREPG plasmid (modified) was used for the *hmgR* gene expression, which was as a gift of Dr. Carr from University of Sussex (England). DH10B was grown at 37 °C in LB liquid medium (10 g l^{-1} oxoid-tryptone, 5 g l^{-1} oxoid-yeast extract, 5 g l^{-1} NaCl) or with 2% Difco Bacto Agar on solid medium, supplemented with 100 μg ml^{-1} ampicillin in selective medium to maintain the plasmids. The fission yeast *Leu*$^{-}$ strain SPQ01 was also kindly provided by Dr. Xia. Yeast cells were grown at 30 °C in YES (5 g l^{-1} yeast extract, 30 g l^{-1} glucose, supplements of 50–225 mg l^{-1} of adenine, uracil, histidine, lysine hydrochloride), and the mutant yeast were grown on EMM minimal media (according Forsburg lab manual). The inducing concentration of thiamine was 15 μM.

Preparation of Genome DNA

Single yeast colony was picked up from streaked agar plates. Yeast cells were inoculated in YES culture at 30 °C and harvested in the late log phase, and the chromosomal DNA was isolated by the genomic DNA extraction Kit (Tiangen, China).

Cloning of *hmgR* Gene

The plasmid pREPG is a shuttle vector [13]. It contains autonomous replication sequence (*ars1*), which is responsible for plasmid maintenance, nmt1 promoter, transcriptional terminator, and *LEU2* a selectable auxotrophic marker. The forward primer sequence was h1, 5′-GGGGGTCGACAATGATTTATAAACTTGCTGCTCGG-3′ harboring S*al*I site, and

the site also followed the start codon region. The reverse primer sequence was h2 5′-TGGGAGATCTATCAACGTCCCGGTACTCG-AGAGTT-3′ containing B*gl*II site and harboring the stop codon region of *hmgR* gene. These oligonucleotides were synthesized in the Applied Biosystems DNA Synthesizer at Invitrogen Beijing Co., Ltd. Polymerase chain reaction (PCR) program was performed on a Thermocycler (Bio-Rad MyCycler, USA), and consisted of: 1 cycle at 94 °C for 4 min, 30 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 3.5 min, as well as a final extension step of 72 °C for 20 min. PCR products were analyzed by 0.8% (*w*/*v*) agarose gel electrophoresis. After purification by a gel extraction kit (Omega), the *hmgR* gene was prepared for construction subsequently.

Construction of Expression Vector for Yeast

The *hmgR* gene and vector pREPG were digested with S*al*I and B*gl*II, respectively. The resulting DNA fragments, after purification from agarose gel, were ligated with each other into the corresponding sites. The recombinant plasmid pREPG was transformed into the competent cells of *E. coli* DH10B. On LB agar plate containing 100 μg ml^{-1} ampicillin, colonies were selected as positive transformants and were identified by colony PCR and further confirmed by plasmid digestion with restriction enzyme. The nucleotide sequence of the *hmgR* gene was verified by the dideoxyribonucleotide chain termination method at Invitrogen (Beijing) Co., Ltd.

Transformation into *S. pombe* and Screening

Screening and confirmation of expressing vectors pREPG with the *hmgR* gene were transformed into *S. pombe* by electroporation [14], and transformants were selected on EMM minimum nutrient agar plates without leucine at 30 °C for 5–7 days, streaking single colony from the selected plates and further shaking them in EMM culture up into late log phase. Collecting 5 ml yeast culture and extracting plasmid from them, transformating plasmid into *E. coli* DH10B again, extracting plasmids from the *E. coli* DH10B transformant further, the correct constructed plasmids were verified by PCR and digestion with enzyme.

Transcriptional Expression Analysis of *hmgR* Gene in *S. pombe*

Total RNA was extracted from fresh yeast cells [15] and removed DNA with DNase I (RNase Free). The reverse transcriptional reaction mixture contained 4.0 μl total RNA (1.0 μg) which was quantified by nanodrop (ND-1000, USA), 1.0 μl dNTP (2 mM), 1 μl oligo (dT)18 (10 mM), 8.5 μl RNase-free water. After incubation of the mixture at 70 °C for 5 min, it was cooled on ice for 2 min, further adding 4.0 μl 5× buffer, 1.0 μl DTT and 0.5 μl reverse transcriptase up to 20 μl. The reverse transcription was carried out at 50 °C for 60 min and terminated at 70 °C for 15 min. The reverse transcription products were stored at −20 °C. PCR amplification was performed on a ThermalCycler (Bio-Rad MyCycler, USA). The reverse transcription products 1 μl were subjected to amplification in a 50-μl mixture containing 0.5 μl Taq DNA polymerase, 5.0 μl 10× PCR buffer, 2.0 μl dNTP, 4.0 μl RT-h1 and RT-h2 (Table 1), and 37.5 μl ddH2O. PCR conditions were: 95 °C for 2 min (1 cycle); 95 °C for 30 s, 50 °C for 30 s, 72 °C for 50 s (less than 30 cycles). *HmgR* gene primers and histone primers for reverse transcriptase PCR (RT-PCR) were synthesized at Invitrogen (Beijing) Co. Ltd. The oligonucleotide sequences were shown as Table 1 with histone gene as an internal control.

Table 1 *HmgR* gene primers and histone primers for RT-PCR system.

Primers were designed at	Oligonucleotides
RT-h1	5′-GGGGTTTTGAATGCATGTTG-′
RT-h2	5′-CAGCAGGCTTCTTATCTGTA-′
His1	5′-ATCTGGAGGTAAGGCCGCA-′
His2	5′-AGCTCTTGGCTAGGCTTGCC-′

Shaking Flask Cultivation of the Recombinants with Induction of Arachidonic Acid

The recombinant cells with pREPG plasmid were as control and the selected recombinant cells with pREPH*mgR* were grown to mid-exponential phase in minimal medium supplemented with 15 μM thiamine at 30 °C, 180 rpm. These cell groups were depressed by thiamine. Parts of these kinds of cells were washed three times with the EMM minimal medium without thiamine to remove the pressure of the nmt1 promoter, and they were further cultured at 30 °C in EMM medium. Cell groups were induced without thiamine. Both of two groups were co-cultured with 50 mg l^{-1} arachidonic acid. And, after growing up to 84 h, pellets were collected to detect the CoQ10 contents. The cells with empty vector, growing on EMM with thiamine depression, were used as the control.

Extraction and Measurement of CoQ10

CoQ10 extracted from *S. pombe* recombinants were analyzed by the method of Zhang et al [16] with modifications. Cells treated by 3 M HCl were stirred at 90 °C for 80 min. After addition of NaOH solution to maintain the pH at 7, the cell pellet was extracted at room temperature for 2.5 h with acetone. The mixture was centrifuged and the upper layer of the solution was vacuum evaporated at 30 °C to evaporate to dryness. It was redissolved in petroleum ether. The CoQ10 was analyzed by HPLC (Shimadzu, Japan) on a C18 reverse-phase column (250×4.6 mm, Dikma) with methanol-hexane (80:20, *v*/*v*) as the mobile phase at a flow rate of 1.5 ml min^{-1}. The inject volume was 15 μl with UV detection at 275 nm and column temperature was set at 30 °C.

Extraction and Measurement of Ergosterol

Dried cells were transferred into a 50-ml flask followed by 4 g of KOH and 16 ml of 60% (*v*/*v*) ethanol/water. The mixture was saponified at 80 °C in water bath for 2 h. Ten milliliters of petroleum ether was added to extract the ergosterol [17]. Ergosterol was quantified by HPLC equipped with a Diamonsil C18 column (250×4.6 mm) at 30 °C using methanol/water (97:3, *v*/*v*) as the mobile phase at 1.8 ml min^{-1}. The inject volume was 15 μl with UV detector at 280 nm.

Results and Discussion

Cloning of the *hmgR* Gene and Construction of Overexpression Plasmid

S. pombe is a valuable alternative model system for studying biological processes since it possesses many properties that closely resembles to those of higher eukaryotes [13, 18]. A

wide variety of specific plasmids of fission yeast have been developed to facilitate molecular manipulation.

In this study, one of pREP series was used as the expression vector, which contains ars1, nmt1 promoter, and *LEU2* as the selectable marker. The nmt1 promoter is repressed by the presence of thiamine in the media. During shake flask culture, induction required minimal media because rich media with yeast extract contain sufficient thiamine to repress the promoter. In this paper, the *hmgR* gene which encodes HMG-CoA reductase was successfully amplified by PCR using genomic DNA of *S. pombe* as template. And, it was cloned into a shuttle vector and located at the downstream of the thiamine repressible nmt1 promoter. DNA sequence analysis revealed that the *hmgR* gene was composed of 3,162 base pairs without introns, starting with ATG and terminating with TGA and encoding 1,053 amino acid residues. Blasting the *hmgR* gene sequence in Genbank, the identity was 99.9% with the sequence (No. L76979) encoding HMG-CoA reductase in *S. pombe*. The construction of overexpression plasmid pREPH*mgR* was shown in Fig. 2.

Transcriptional Expression Analysis of *hmg*R Gene

To investigate the transcriptional expression level of *hmgR* gene, a series of yeast DNA bands were identified by an approach of quantitative RT-PCR. Different specific primers summarized in Table 1 were used to check the transcriptional level of *hmgR* gene in recombinant yeast cells, and the histone gene, using as the internal control, could be detected with 25 and 27 cycles. The gene contents were based on the band light shown in Fig. 3. The results showed that it could be detected transcriptional messages of the *hmgR* gene amplified by the pair of primers RT-h1 and RT-h2 at 25 cycles. No signals were presented in yeast with pREPG plasmid in EMM culture without thiamine, but transcripts of *hmgR* gene can be detected in yeast with pREPH*mgR* plasmid under the same growth condition. The detectable signal in pREPH*mgR* yeast was notably stronger, adding arachidonic acid into EMM culture. When PCR was performed with 27 cycles, histone genes should be given higher bands. *HmgR* gene can be detected in yeast with pREPG plasmid. According to the DNA bands light, we could conclude that the mRNA level of *hmgR* gene was higher in pREPH*mgR* yeast than in control yeast. This result showed that upregulation of HMG-CoA reductase was confirmed in pREPH*mgR* yeast. However, the mRNA level of *hmgR* gene of yeast in EMM culture with arachidonic acid was higher than that without arachidonic acid, so it is deduced that arachidonic acid could significantly stimulate HMG-CoA reductase to show a higher transcriptional level.

Effect of *hmgR* Overexpression on Ergosterol and CoQ10 Contents

Isoprends are the same biosynthesis precursors of ergosterol and CoQ10. Previous studies have indicated that HMG-CoA reductase is a key enzyme involved in ergosterol biosynthesis [1]. To detect the effect of HMG-CoA reductase in *S. pombe*, the cells with pREPH*mgR* plasmid were employed in this experiment, using the pREPG yeast as a

Fig. 2 Expression cassettes of *hmgR* gene was designed. S*al*I and B*gl*II were two cloning sites. Pnmt1 and Tnmt1 are the promoter and terminator of nmt1, respectively. *LEU2* is the selective marker

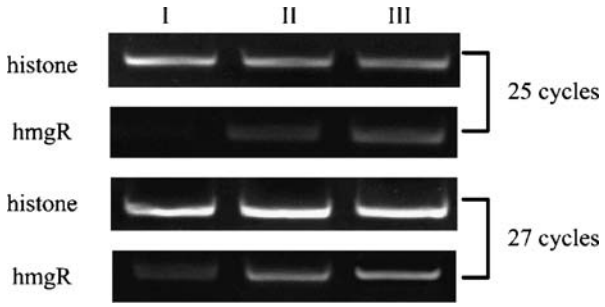

Fig. 3 RT-PCR of *hmgR* gene in *S. pombe*. The mRNA was detected using quantitative RT-PCR with histone as an internal control. Histone bands were 441 bp which performed by His1 and His2 primers. *HmgR* bands were 527 bp which performed by RT-h1 and RT-h2 primers. LaneI RNA sample were extracted from pREPG yeast. LaneII RNA samples were extracted from pREPH*mgR* yeast. LaneIII RNA samples were extracted from pREPH*mgR* yeast which grow with co-cultivation of arachidonic acid

control. The growing condition of yeast was described in "Strains, Plasmids, and Culture Conditions". There were no difference in growth pattern of yeast cells between pREPG yeast and pREPH*mgR* yeast (data not shown). Fresh yeast cells were collected at 84 h, and CoQ10 contents were analyzed by HPLC. All values in Table 2 are means of 3 time experimental results. In stationary phase, the CoQ10 contents in pREPH*mgR* cells were almost the same as that in pREPG strains under the depression of nmt1 promoter. After removal of thiamine from the culture, the maximal CoQ10 contents of pREPH*mgR* strain, carrying full-length gene, were 2.68 times than that of control yeast. Interestingly, arachidonic acid was added at the beginning of pREPH*mgR* yeast growth, CoQ10 contents in pREPH*mgR* yeast increased significantly (3.09 times), while that in pREPG yeast enhanced a little (1.41 times). It showed some increase trends on the ergosterol contents of the cells. The ergosterol contents in pREPH*mgR* yeast with arachidonic acid co-cultivation increased much higher than in pREPH*mgR* yeast (Table 2). In this study, the results indicated that HMG-CoA reductase accumulation could improve CoQ10 and ergosterol contents, because both ergosterol and CoQ10 share the mevolonate pathway.

Effect of Arachidonic Acid to HMG-CoA Reductase

In the RT-PCR reaction, the mRNA transcriptional level of the *hmgR* gene, in pREPH*mgR* yeast with arachidonic acid co-cultivation, drastically increased more than that without co-cultivation. The HPLC analysis results showed that arachidonic acid has increasing effect on the contents of CoQ10. There were more CoQ10 contents in pREPG yeast after

Table 2 CoQ10 and ergosterol contents of *S. pombe* carrying *hmgR* gene mutant and induction with arachidonic acid.

	pREPG			pREPH*mgR*		
	Depress	Induce	AA[a]	Depress	Induce	AA[a]
Ergosterol (mg g^{-1})	24.1	25.7	29.6	25.8	39.1	44.1
CoQ10 (mg g^{-1})	0.22	0.20	0.31 (1.41 times)	0.25	0.59 (2.68 times)	0.68 (3.09 times)

[a] Represented incubation with arachidonic acid

treatment with arachidonic acid. Furthermore, in the *hmgR* gene overexpressing strains, pREPH*mgR* yeast cells were also stimulated to keep much higher CoQ10 level in the same co-cultivating condition.

It was also reported that plant cells were sensitive to arachidonic acid, which could increase one of HMG-CoA reductase family activity in plant cells [11]. In this experiment, fission yeast was employed, which possessed only one *hmgR* gene. Arachidonic acid, a special kind of polyunsaturated fatty acids, could still stimulate the transcriptional of HMG-CoA reductase. CoQ-deficient budding yeast also showed hypersensitive to polyunsaturated fatty acids [12]. While in CoQ10-overexpressed yeast cells, arachidonic acid can promote the improvement of cells growth and intracellular CoQ10 contents. The possible reason was that arachidonic acid can eliminate free ions and acts as an antioxidant in *S. pombe*. In addition, maximal CoQ10 yield was detected after adding arachidonic acid into pREPH*mgR* yeast culture, while it also increased in control yeast. It indicated that arachidonic acid could stimulate the HMG-CoA reductase to have a higher transcriptional level after overexpression of *hmgR* gene, then catalytic efficiency increased. However, the mechanism of arachidonic acid stimulating the transcriptional level of HMG-CoA reductase is still unclear. It will be an interesting work to investigate the possible reasons further. With the increasing demand for CoQ10, investigation of biosynthesis pathway of CoQ10 in yeast is a very important subject. HMG-CoA reductase was considered as a key enzyme of CoQ10 biosynthesis. However, the increase of ergosterol contents resulted from HMG-CoA reductase accumulation, which branched some of carbon fluxes. Therefore, inhibiting metabolic flux into sterol is a promising research topic to improve metabolic flux of CoQ10 further.

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