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Short communication

Impact of methyl jasmonate on squalene biosynthesis in microalga *Schizochytrium mangrovei*

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ABSTRACT

Squalene is an effective antioxidant and a potential chemopreventive agent. In this work, the effect of methyl jasmonate (MJA) on squalene biosynthesis in microalga *Schizochytrium mangrovei* was investigated. The maximum squalene content (1.17 ± 0.06 mg/g cell dry weight, DW) reached during the next 3 h after MJA treatment (0.1 mM) at 48 h of cultivation, which was 60% higher than that of control. The activity of squalene synthase (SS) increased 2-fold over control at this point. The maximum cholesterol content of 0.45 ± 0.03 mg/g DW was reached at hour 51 when MJA concentration was 0.4 mM, whereas the squalene content was lower at this point. The observations suggested that the increased squalene content was resulted from an increased activity of SS. MJA could be used to regulate the key enzymes in squalene biosynthetic pathway for the increased production of this compound in thraustochytrids. This research also provided novel information on the stimulation effect of methyl jasmonate on the biosynthesis of essential intermediate involved in the primary metabolism in microorganism.

1. Introduction

Squalene is an important intermediate in the cholesterol biosynthetic pathway. It is an essential natural antioxidant to protect the cells from free radicals and reactive oxygen species. It plays a major role in releasing oxidative stresses, such as sunlight exposure [1]. Squalene is also effective in decreasing serum cholesterol level and has cardioprotective effect on myocardial infarction in experimental animals [2,3]. Experiments *in vitro* and in animal models have shown that squalene can effectively inhibit colon, lung and skin tumourigenesis in rodents. In some regions, squalene is a popular folk medicine for chronic liver diseases [4]. Squalene has been regarded as an effective chemopreventive agent and has the great potential to be used in clinical trial [5–7].

The traditional sources of squalene are liver oils of deep-sea sharks and whales. Large-scale extraction of squalene from marine animals, however, is nearly impossible as a result of international concern over the protection of endangered marine animals [8]. Furthermore, although the application of squalene in cosmetics is in great quantities, some leading cosmetic companies are phasing out the use of shark liver oil and other animal-based squalene in their products in response to the growing pressure from the environmental campaign. The potential increasing demand for squalene therefore, has led to a worldwide interest in searching for other novel and sustainable sources of this compound. Recently we found the microalga—*Schizochytrium mangrovei*, a unicellular thraustochytrid could be used as potential producer of squalene [9]. Because microbial production of squalene using fermentation technology has been regarded as the most cost-effective strategy for the stable commercial supply of this useful product. To further increase the squalene content of this microalga, in addition to optimize the environmental culture conditions, the metabolic regulation on the activities of key enzymes in the squalene biosynthetic pathway is worthy of investigation.

Jasmonates and its methyl ester methyl jasmonate (MJA) are always considered as potent lipid regulators that modulate various physiological processes in plants, such as growth, senescence, reproduction and responses to both mechanical trauma and pathogenesis [10,11]. Up to now, MJA has been successfully applied to induce or increase the biosyntheses of many important secondary metabolites in plant cell, e.g. ginsenoside and paclitaxel [12–15] that have been widely used by human beings as drugs and nutraceuticals. However, there has been no such report on the effect of methyl jasmonate on the activities of the key enzymes in unicellular microalga for the production of useful metabolite. Moreover, the information on the effect of methyl jasmonate on the biosynthesis of essential intracellular intermediate involved in the primary metabolism is very limited.

In squalene biosynthetic pathway, acetoacetyl-CoA generated through glycolysis is used to synthesize 3-hydroxy-3-methylglutaryl-CoA, which is further converted to farnesyl diphosphate

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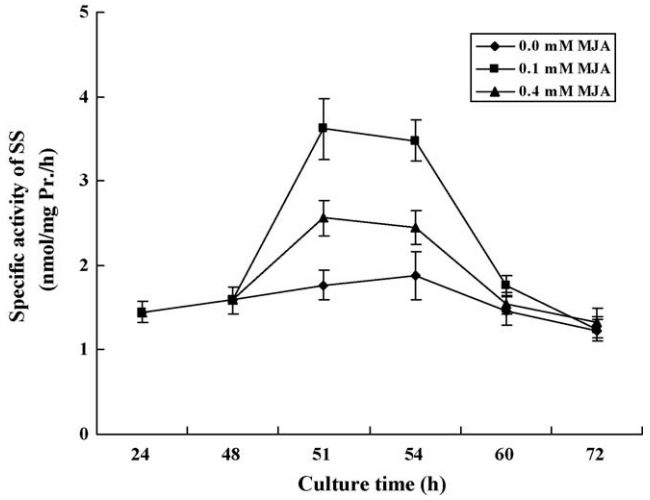

Fig. 1. Responses of squalene synthase (SS) of *S. mangrovei* to MJA addition at cultivation hour of 48. Symbols: (◆) 0 mM MJA; (▲) 0.1 mM MJA; (■) 0.4 mM MJA.

through several steps. Squalene is then synthesized directly from farnesyl diphosphate. In cholesterol biosynthetic pathway, squalene is the last metabolite preceding sterol ring formation [16]. Within the steps, squalene synthase (EC 2.5.1.21, SS), which catalyzes a reductive dimerization of two farnesyl diphosphate (FPP) molecules into squalene, represents a putative branch point in the isoprenoid biosynthetic pathway diverting carbon flow specifically to the biosynthesis of sterol and triterpene. It is therefore considered as a key regulatory point for squalene metabolism. However, no such information is available on the regulation of SS for squalene biosynthesis in thraustochytrids.

In this study, the responses of *S. mangrovei* to the addition of MJA for squalene biosynthesis were investigated. The content of squalene, the specific SS activity and cholesterol content in *S. mangrovei* under different conditions were analyzed, because cholesterol is one of the major sterols of thraustochytrids for cell growth [17]. Our work aimed to investigate the effectiveness of MJA on squalene biosynthesis in *S. mangrovei* in order to propose a proper strategy to increase the production of squalene by this microalga. The possibility of using enzyme stimulator on the large amount accumulation of the indispensable intermediate (i.e. squalene) whose metabolites are essential for cell growth would be evaluated. Moreover, the result from this study will also provide important information on the application of plant elicitor—MJA on the enhanced biosynthesis of primary metabolite in microalgae.

2. Materials and methods

2.1. Heterotrophic growth of S. mangrovei

S. mangrovei used in this study was isolated from decaying *Kandelia candel* leaves from local mangroves in Sai Keng, Hong Kong and maintained on agar slant according to the methods described by Fan et al. [18]. The inoculum and culture were prepared in 250-mL Erlenmeyer flasks (each containing 100 mL of medium) and incubated at 22 °C in an orbital shaker at 120 rpm in the dark. The medium composition was same as that described in Jiang et al. [9]. The inoculum volume was 5% (v/v).

2.2. Methyl jasmonate (MJA) treatment

MJA (0.1 M) was dissolved in dimethyl sulfoxide (DMSO) and sterilized by passing through 0.22 μm polyvinylidenedifluoride (PVDF) syringe filter (Millipore) before adding to the cell culture. The culture added with same amount of DMSO without MJA was used as control. Each treatment was repeated for three times. Data were expressed as mean plus standard deviation of triplicates.

2.3. Determination of cell dry weight (DW)

The cell dry weight was determined according to Fan et al. [18].

2.4. Analyses of squalene and cholesterol contents

The cellular squalene and cholesterol contents were analyzed according to Lu et al. [19] and Saldanha et al. [20] with some modifications. Freeze-dried cells (100 mg) were saponified by 4 mL of 10% (w/v) KOH-75% (v/v) ethanol solution at 50 °C for 15 min. The mixture was extracted with 4 mL of hexane for three times. The hexane layer was collected and evaporated to dryness under N_2. The residue was dissolved in 2.0 mL of acetonitrile for subsequent HPLC analysis. Waters 2695 HPLC (Waters, Milford, MA, USA) equipped with a Waters 2996 photodiode array detector and a reversed-phase Superspher C18 column (150 × 4.0 mm i.d. 5 μm, Merck) were used. The separation of squalene and cholesterol was achieved by using a mobile phase of 100% acetonitrile at a constant flow rate of 1.5 mL/min and a mobile phase of 100% methanol at a constant flow rate of 1.0 mL/min, respectively. The squalene and cholesterol were monitored at wavelength of 195 and 205 nm, respectively. The peaks of squalene and cholesterol were identified based on their retention time and UV spectra against those obtained from squalene and cholesterol standards, respectively (Sigma Chemical Co., USA). The quantification was done according to the external calibration graphs obtained from the peak areas vs. different concentrations of squalene and cholesterol standards, respectively. All samples and standards were filtered through 0.45 μm membrane filters before injection.

2.5. Preparation and assay of squalene synthase (SS)

The assay was performed as described by Okada et al. [21] with some modifications. Briefly, the cell pellets harvested from 100 mL broth were suspended in 45 mL of solution A consisting of 50 mM Tris–HCl (pH 7.2), 5 mM 2-mercaptoethanol and 20 mM $MgCl_2$. The suspension was centrifuged (4000 × *g*) at 4 °C for 10 min. The cell pellet (1 g) was frozen by liquid nitrogen and homogenized in ice bath with 5 mL of extraction buffer consisting of 250 mM Tris–HCl buffer (pH 7.2), 250 mM sucrose, 5 mM 2-mercaptoethanol, 20 mM $MgCl_2$ and 1 mM PMSF. The homogenate was centrifuged at 5000 × *g* for 10 min at 4 °C. The supernatant was collected and filtered through three layers of gauze. The filtrate was used as enzyme extract for subsequent SS assay. The reaction mixture (0.2 mL) consisted 0.15 mL of solution B (250 mM Tris–HCl buffer (pH 7.2), 10 mM NADPH, 10 mM $MgCl_2$, 2.5 mM 2-mercaptoethanol, 0.23 mM farnesyl diphosphate) and 0.05 mL of enzyme extract. The one without the addition of farnesyl diphosphate was used as blank. The reaction was kept at 35 °C for 30 min and terminated by adding 0.4 mL n-hexane followed by mixing and centrifugation at 15,000 × *g* for 3 min. The hexane was collected and the residue reaction mixture was extracted with 0.4 mL hexane twice. The hexane layers were combined and evaporated to dryness under N_2. The residue was re-dissolved in 0.04 mL of acetonitrile for squalene analysis as indicated above. The amount of squalene converted from farnesyl diphosphate catalyzed by SS was obtained by subtracting the squalene content of the blank from that of the reaction mixture with the addition of farnesyl diphosphate. The specific activity of SS was expressed as nmol squalene/mg protein (Pr)/h. The protein content was measured by Bradford method [22] with bovine serum albumin (Sigma Co., USA) as standard.

3. Results and discussion

3.1. Influence of MJA on the growth and squalene biosynthesis in S. mangrovei

As no effect of DMSO (ranging from 0.05 to 0.4 mL in every 100 mL culture broth) on the growth, squalene and cholesterol contents as well as the activity of SS in *S. mangrovei* was observed (data not shown), DMSO was selected as the solvent to dissolve MJA in this study. Effects of MJA on the growth and squalene biosynthesis of *S. mangrovei* were investigated by adding MJA ranging from 0 to 0.4 mM after 48-h cultivation (Tables 1 and 2). For cell growth, the cell dry weight (DW) at hours 60 and 72 were apparently lower than that of control when the concentration of MJA was 0.4 mM. But no obvious difference was observed when the concentration of MJA ranged from 0.05 to 0.2 mM (Table 1). The squalene content was greatly increased after low concentration MJA treatment but decreased when MJA concentration was high (Table 2). The maximal squalene content of 1.17 ± 0.06 mg/g DW was reached, which was 1.6-fold of that of control, during the next 3 h after MJA (0.1 mM) treatment at cultivation hour of 48.

During MJA treatment, squalene content in *S. mangrovei* increased rapidly (60% increment compared to the control) after the treatment of 0.1 mM MJA at 48 h. But the increment could only be maintained for a short period of time. This might be due to the