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Biosynthesis of 4-acetylantroquinonol B in *Antrodia cinnamomea* via a pathway related to coenzyme Q synthesis

Shang-Han Yang, Yu-Wei Lin, Been-Huang Chiang*

Institute of Food Science and Technology, National Taiwan University, No.1, Roosevelt Road, Section 4, Taipei, Taiwan, ROC

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ABSTRACT

The biosynthesis pathway for production of 4-acetylantroquinonol B (4-AAQB) by *Antrodia cinnamomea* was investigated by adding various precursors to the culture medium. Adding 4-hydroxybenzoic acid (4-HBA) significantly increased the production of 4-AAQB. Since 4-HBA is an intermediate of the shikimate pathway and 4-AAQB and coenzyme Q (CoQ) are similar in structure, we suspected that the pathway for producing 4-AAQB was closely related to the biosynthesis of CoQ. Since the isoprenoid chain of CoQ is synthesized via the mevalonate pathway, we added oleic acid to the culture medium and confirmed that the addition significantly increased the production of 4-AAQB. Furthermore, adding coenzyme Q_0 into the fermentation broth was found to be the most effective way to increase the production of 4-AAQB. We suspect that coenzyme Q_0 forms CoQ, after which CoQ is converted to 4-AAQB via unknown steps. The increase in 4-AAQB production due to the addition of CoQ_{10} further demonstrated that the biosynthesis pathway of 4-AAQB from *A. cinnamomea* is closely related to CoQ.

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1. Introduction

Antrodia cinnamomea is a well-known medicinal fungus often produced commercially by submerged fermentation in Taiwan. This fungus is known for its potent anti-hepatocellular carcinoma function. In a previous study, the key anti-hepatic compound from the mycelium of *A. cinnamomea*, 4-acetylantroquinonol B (4-AAQB), was isolated and identified. This compound can inhibit proliferation of hepatocellular carcinoma cells HepG2 with an IC_{50} of 0.1 μg/mL [1]. When the HepG2 cells were treated with 4-acetylantroquinonol B, CDK2 and CDK4 decreased and p27 increased in a dose-dependent manner [2]. The treatment also increased the levels of p53 and p21 proteins. A previous study found that in the biosynthesis of 4-acetylantroquinonol B [3], based on the biosynthesis pathway of coenzyme Q, the benzoquinone ring might be produced from shikimic acid via the shikimate pathway and that the polyisoprenoid side chain may be produced from farnesyl diphosphate via the mevalonate pathway. Furthermore, the structure of 4-AAQB is very similar to that of coenzyme Q, so we suspected that the biosynthesis pathway of 4-AAQB was closely related to Coenzyme Q.

Previous reports have discussed the biosynthesis of coenzyme Q. For *Saccharomyces cerevisiae*, the benzoquinone ring is formed from 4-hydroxybenzoic acid (4-HBA), which can be synthesized from chorismate via the shikimate pathway [4]. In *E. coli*, the aromatic ring also comes from 4-HBA, and nine enzymes involved in modification of the ring [5]. Recent research on the overproduction of coenzyme Q_{10} in microorganisms has been extensive [6]. Throughout the pathway, the exogenous addition of correct precursors to the medium, and overexpression of various enzyme genes are strategies for improving the yield of coenzyme Q_{10}. We proposed that in *A. cinnamomea*, 4-AAQB was synthesized via the biosynthesis pathway of CoQ, after which CoQ would be converted to 4-AAQB. Various precursors of CoQ were tested in this study in order to construct the possible biosynthesis pathway of 4AAQB in *A. cinnamomea* (Fig. 1).

2. Materials and methods

2.1. Microorganism and reagents

Antrodia cinnamomea BCRC35716 was obtained from the Bioresoures Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Potato dextrose agar (PDA), malt extract, and peptone were obtained from Difico (Sparks, MD, USA). 4-hydroxybenzoic acid (4-HBA), 2, 3-dimethoxy-5-methyl-*p*-benzoquinone (coenzyme Q_0), coenzyme Q_{10}, L-phenylalanine (Phe), tyrosine (Tyr), vanillic acid (VA), 2, 4-dihydroxybenzoic acid (2,4-DHBA), 4-aminobenzoic acid (4-ABA)

* Corresponding author.
E-mail address: bhchiang@ntu.edu.tw (B.-H. Chiang).

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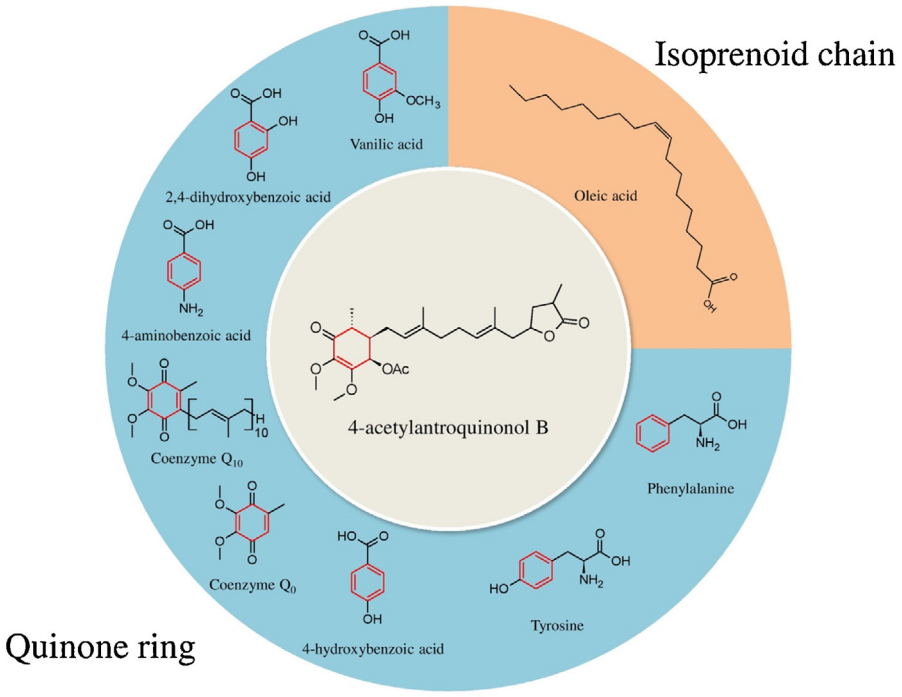

Fig. 1. Precursors for 4-acetylantroquinonol B.

and oleic acid were obtained from Sigma-Aldrich (St. Louis, Mo, USA).

2.2. *Shake-flask fermentation of* Antrodia cinnamomea

The seed culture was maintained on 39 g L^{-1} potato dextrose agar at 25 °C and transferred to a fresh agar plate every 4 weeks. To prepare the inoculum, the mycelium of *A. cinnamomea* was transferred from the Petri dish to a 500 mL flask containing 200 mL of medium (components: glucose 2.0%, malt extract 2.0%, and peptone 0.1%) and incubated at 25 °C for 7 days for mycelium growth. Then, 20 mL of the flask culture was transferred to a 500 mL flask containing 200 mL medium. The mixture was fermented at 25 °C for 4 weeks in a rotary shaker at 100 rpm. The initial pH of the medium was 5 adjusted by 0.1 N NaOH or 0.1 N HCl.

The *A. cinnamomea* was cultivated in the medium described above with 0.01% phenylalanine, tyrosine, coenzyme Q_0, coenzyme Q_{10}, vanillic acid, 2, 4-dihydroxybenzoic acid, 4-aminobenzoic acid and 0.01, 0.02, 0.05 or 0.1% 4-HBA. Precursors were combined in the same medium with both 0.01% 4-HBA and coenzyme Q_0 or 0.1% oleic acid added. The pH value of the medium was adjusted to 5 by adding 0.1 N NaOH or 0.1 N HCl, and then the mixture was sterilized at 121 °C for 20 min.

2.3. *Determination of biomass and 4-acetylantroquinonol B content*

To recover the mycelium, the fermentation broth was through Whatman No. 1 filter paper and the filtrate was washed twice with distilled water. Biomass was then determined after freeze-drying. The ethanol extract of the mycelium of *A. cinnamomea* was obtained by extracting freeze-dried mycelium (0.1 g) with 95% ethanol (2 mL) by sonication at 25 °C for 1 h. The extract was centrifuged at 25 °C for 1 h at 10,000 rpm. The supernatant was filtered through 0.45 μm membrane and then the concentration of 4-AAQB was analyzed by Agilent 1100 HPLC system (Agilent, USA) equipped with an UV detector. A COSMOSIL $5C_{18}$-MS-II column (4.6mm × 250 mm, 5 μm) was used for separation. The mobile phase was composed of H_2O (A), 0.1% phosphate buffer:methanol = 7:13 (B) and acetonitrile (C). The elution profile was as follows: 0–45 min, B 100%; 45–60 min, A:C = 90:10; 60–75 min, A:C = 10:90; 75–90 min, B 100%; the flow rate was 1 mL/min and the detection wavelength was set at 254 nm. Pure 4-AAQB isolated from the mycelium of *A. cinnamomea* in a previous study (Lin et al., 2011) [2] was diluted to 31.25, 62.5, 125, 250, 500 and 1000 μg/mL for constructing the standard curve (R^2 = 0.9993).

2.4. *Statistical analysis*

Significant differences among means ($p < 0.05$) were determined by one-way analysis of variance and Duncan's multiple-range test (SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

The biosynthesis pathway of CoQ is different in different species (Fig. 2). Yeast and fungi can make the benzoic ring from 4-HBA, and yeast is also able to derive the ring of CoQ from the folate precursor *para*-aminobenzoic acid (*p*ABA) [7]. In vertebrates, mammals can incorporate phenylalanine and tyrosine into the benzenoid ring of CoQ, and 4-hydroxybenzoic acid is a possible intermediate [4]. Since the structure of 4-acetylantroquinonol B is very similar to that of CoQ, in this study, we used some of the important intermediates in CoQ synthesis as precursors to determine whether these intermediates also play significant roles during the synthesis of 4-AAQB, so that we could try to construct the biosynthesis pathway of 4-AAQB.

3.1. *Effect of aromatic amino acid on the biosynthesis of 4-acetylantroquinonol B*

In the biosynthesis of CoQ in *E. coli*, 4-HBA is formed from shikimate via chorismate. Research shows that *S. cerivisiae* forms 4-HBA via two different pathways [8]. In yeast cells, 4-HBA can

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