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Enhancement of cordycepin production in submerged cultures of *Cordyceps militaris* by addition of ferrous sulfate

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ABSTRACT

The effect of ferrous sulfate addition on production of cordycepin (3′-deoxyadenosine), a useful bioactive product with some pharmacological activities, was investigated in submerged cultures of *Cordyceps militaris* in shake flasks. The results showed that the optimal addition condition was on day 0 with 1 g/L of ferrous sulfate, and the maximal amount of cordycepin reached 596.59 ± 85.5 mg/L, about 70% higher than the control without ferrous sulfate addition. Meanwhile, the consumption of inosine 5′-monophosphate (IMP), a potential precursor of cordycepin, was decreased rapidly. Transcription levels of important genes encoding adenylosuccinate synthetase (*pur*A), IMP cyclohydrolase (*pur*H) and IMP dehydrogenase (*gua*B) in the purine nucleotide biosynthetic pathway were also studied. Compared to the control, the transcription level of *pur*A was significantly up-regulated in ferrous sulfate supplemented cultures, while *pur*H and *gua*B were slightly down-regulated. This work indicated that ferrous sulfate addition was a simple and useful strategy for improving cordycepin production, and the related information might be helpful for further manipulation and understanding of the cordycepin biosynthesis.

1. Introduction

Fungi are a valuable source for biotechnology application and have been receiving great interest [1,2]. *Cordyceps militaris* is an entomopathogenic fungus, which is widely used as a popular tonic and traditional Chinese medicinal mushroom. It produces various bioactive compounds, among which cordycepin (3′-deoxyadenosine) is a major active one [3]. As an adenosine analogue, cordycepin has been reported to exhibit some significant pharmacological functions, including immunomodulatory, anti-inflammatory, anti-tumor, anti-metastatic and anti-bacterial activities [4].

Until now, the cordycepin biosynthetic pathway has not been completely elucidated. Previous studies on the incorporation of various ^{14}C-labeled compounds (adenosine, adenine, glucose, etc.) and ^{3}H-labeled ribose into cordycepin showed that most of them might serve as potential precursors [5,6]. Cordycepin is considered to be synthesized via purine nucleotide pathway since the purine moieties are the same. As shown in Fig. 1, the *de novo* purine nucleotide pathway involves the sequential conversion of phosphoribosyl pyrophosphate (PRPP) to IMP and then to AMP and GMP. It has been established that phosphoribosylamidotransferase (PRAT) is the rate-limiting enzyme of the central purine nucleotide pathway; the enzyme IMP cyclohydrolase (IMPC) catalyzes the last step specific to IMP biosynthesis [7]; IMP dehydrogenase (IMPDH) catalyzes the first enzymatic step of IMP oxidation towards GMP biosynthesis [8]; and adenylosuccinate synthetase (AS) governs the committed step specific to AMP biosynthesis from IMP, which shares the same adenine ring skeleton of cordycepin [9].

Because of its strict requirement for growth condition and hosts as well as over harvesting, wild *C. militaris* is considered to have disappeared in nature along with environment change [10,11]. Submerged cultivation is receiving popularity as an alternative to artificial field production. Many researchers have studied various cultivation conditions to improve mycelial growth and cordycepin production since 1960s, such as adding various carbon and nitrogen sources, amino acids, inorganic salts and precursors [12–14]. A two-stage cultivation strategy to cultivate *C. militaris* was also reported [15]. In addition, there were some reports on the effect of trace metals on the cultivation of *C. militaris*. For example, Mn^{2+} at 0.1 mM was identified to stimulate the cell growth and synthesis of some nucleosides like uridine, guanosine, and adenosine [12]. The activity of a novel fibrinolytic enzyme from *C. militaris* was increased by metal ions such as Mg^{2+} and Fe^{2+}, whereas inhibited by Cu^{2+} [16]. Wang et al. [17] observed that addition of a suitable concentration of Mn^{2+}, Zn^{2+} or Cu^{2+} into the medium could induce

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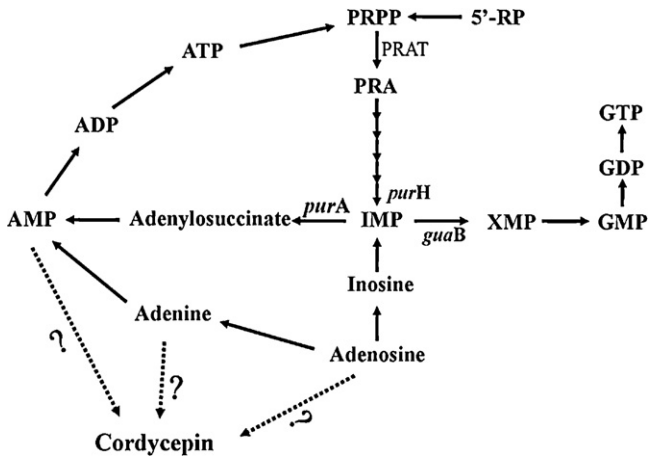

Fig. 1. The *de novo* purine nucleotide pathway and possible cordycepin biosynthetic pathway. Dash lines indicate that those reaction steps are still unknown. *pur*A, adenylosuccinate synthetase gene; *pur*H, IMP cyclohydrolase gene; *gua*B, IMP dehydrogenase gene; PRAT, phosphoribosylamidotransferase.

the production of superoxide dismutase in *C. militaris* mycelium. Iron could also participate as a biocatalyst or electron carrier in numerous biological processes, such as electron transport, nitrogen assimilation, DNA synthesis, respiration and gene regulation [18]. High concentrations of iron make it harmful to organisms and environment and even cause oxidative stress [19]. In *Streptomyces* sp., Fe^{3+} was beneficial for production of neomycin, streptomycin, actinomycin and chloramphenicol [20]. The cell growth was found to be closely related to iron concentration in *hrp*-inducing minimal medium and the expression of several virulence-related genes was also affected by iron availability in *Pseudomonas syringae* pv. *tomato* DC3000 [21]. However, to the best of our knowledge, there have been no reports on the effect of iron on production of cordycepin and its biosynthetic gene transcription.

In this work, the effect of ferrous sulfate addition on cordycepin production was studied in shake flask cultures. Intermediate accumulation and transcription levels of three genes encoding adenylosuccinate synthetase, IMP cyclohydrolase and IMP dehydrogenase in the purine nucleotide pathway were also investigated. The information obtained would be beneficial not only to large-scale production but also to further manipulation and understanding of the cordycepin biosynthesis in future.

2. Materials and methods

2.1. Main chemicals

HPLC-grade methanol was purchased from Labar (Lingfeng Chemical Reagent Co., Shanghai, China). Standard cordycepin from the National Institute for the Control of Pharmaceutical and Biological Products (Shanghai, China) and IMP (purity >98%) from Sigma were used to establish the detection calibration curves. Other chemicals used in this study were from Shanghai Chemical Reagents Co. (China) and of analytical purity.

2.2. Fungi material and culture conditions

The strain of *C. militaris* used in this study was from the collection bank of Huazhong Agricultural University (Hubei, China). Details of culture media and procedures of inoculation were described previously [22] with a modification of cultivation medium containing glucose 40 g/L, peptone 10 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, $K_2HPO_4{\cdot}3H_2O$ 0.5 g/L, KH_2PO_4 0.5 g/L and $(NH_4)_2SO_4$ 2.64 g/L. The cultivation was conducted on a rotary shaker (25 °C, 110 rpm) in the dark with pH 6.0 for 15 or 20 days.

In order to observe the effect of iron on *C. militaris* cultivation, $FeSO_4{\cdot}7H_2O$ was added to a different final concentration (0.1, 1, 2, 3, 4 and 5 g/L) in the cultivation medium. The cells treated with an equal volume of deionized water were used as control. Four different addition points, namely day 0, 4, 6 and 8, corresponding to the early, middle, and late growth stages [13], were conducted. Under the optimal iron addition, the cells were harvested on day 4, 10, 15 and 20 in shake flask cultures. For sampling, three identical shake flasks were used for each data point.

2.3. Measurement of biomass, residual sugar, total iron, IMP and cordycepin in the medium

The culture broth from the shake flasks of *C. militaris* was centrifuged at 10,000 × *g* for 10 min, and the mycelia were washed at least three times with sufficient distilled water through a filter paper and then dried at 60 °C to a constant weight for cell dry weight assay. Part of the supernatant was used for measuring pH, and the remainder was stored at −20 °C for analyzing residual sugar and cordycepin later.

Residual sugar concentration was assayed by a phenol–sulfuric acid method [23]. About 0.5 g dry weight cells were used to measure the total intracellular iron level by an inductively coupled plasma mass spectrometer (ICP-MS) [24]. The content of iron accumulated in mycelia was calculated by the following formula (μmol/g): [iron content in sample mycelia (ppm)/56]. Both IMP (the intermediate) and cordycepin were analyzed by Agilent 1200 series HPLC system (USA) equipped with a vacuum degasser, a quaternary gradient pump, and a diode array detector. Both separations were carried out on a Hypersil ODS2 5 μm RP18 column (4.6 × 250 mm, 5 μm) (Eliter Company, Liaoning, China). As for IMP, 0.1 g fresh cells were extracted with 4 mL of 0.6 M perchloric acid at 0 °C for 1 min and then centrifuged at 3000 × *g* for 10 min. Prior to HPLC analysis, 2 mL of the supernatant was neutralized immediately to pH 6.5–6.8 with 1 M KOH followed by dilution to 4 mL. The eluent was composed of 0.04 M KH_2PO_4 and 0.06 M K_2HPO_4 buffer [25].

The mobile phase for cordycepin analysis was methanol/water (15:85) with 10 mM KH_2PO_4. The supernatant from sampling flasks was centrifuged at 15,000 × *g* for 15 min followed by filtering through 0.22 μm Nylon syringe filters (Rephile Bioscience and Technology, Shanghai, China) before injecting into the system [13]. HPLC analysis was under the following condition: flow rate, 1 mL/min; column temperature, 30 °C; UV wavelength, 254 nm; sample injection volume, 20 μL. The chromatographic peaks were identified by comparing the retention time and spectrum against those of the standards.

2.4. Total RNA extraction and cDNA preparation

Total RNA of about 100 mg fresh cells was extracted with 1 mL Trizol (Invitrogen, Carlsbad, CA, USA). RNA concentration was determined using a Biophotometer Plus (Eppendorf, Germany). DNase I (Fermentas, Lithuania) was used to remove the residual genomic DNA and RevertAid™ M-MuLV Reverse Transcriptase (Fermentas, Lithuania) to reverse-transcribe first-strand cDNA according to the manufacturer's protocol.

2.5. Primer designing and qRT-PCR analysis

Partial cDNA of *C. militaris* 18S rRNA was isolated by polymerase chain reaction (PCR) amplification using primers as follows (Sangon, Shanghai, China): forward, 5′-GTGGCGGTGAAACAGGACTT-3′; reverse, 5′-TCAGCCTTGCGACCATACTC-3′ (AJ009681.1, GI: 5689685). The partial cDNAs of three genes in the purine