Identification of 3’deoxyadenosine (Cordycepin) from the medicinal mushrooms, Ophiocordyceps spp

Sangeetha Chinnusamy and Akkanna Subbiah Krishnamoorthy

Abstract
The antifungal metabolite cordycepin was extracted and purified from cell free culture filtrate condensate and mycelial mat extract of Ophiocordyceps sinensis and Ophiocordyceps neovolkiiana by TLC. The compound exhibited the band with the Rf value of 0.63. Further confirmation by HPLC indicated only from the culture filtrate and mycelial mat extract of O. sinensis and the retention time had ranged between 5.8 - 5.9 min as against 5.8 min with the standard cordycepin molecule (Sigma Aldrich, Inc., USA). Inopportunistly, the native isolate of O. neovolkiiana not had the similar peak at the same retention time. But the proton NMR spectral analysis of cordycepin produced by both, O. sinensis and O. neovolkiiana (CFC filtrate condensate and mycelial mat extract) showed anomeric carbon peaks at 3.4 ppm, which were comparable with proton NMR spectrum of standard cordycepin already done.

Keywords: O. sinensis, O. neovolkiiana, cordycepin, Thin Layer Chromatography (TLC), High performance liquid chromatography (HPLC) and NMR

1. Introduction
The Chinese caterpillar fungi, Ophiocordyceps spp are usually identified based on the fungal growth observed on insect cadavers and they are formulated as inundative biocontrol agents of Lepidopteran insects, mites and ticks (Goettel et al., 2005 and Vincent et al., 2007) [6, 27]. Ophiocordyceps sinensis is known to parasitize the larvae of ghost moth (Hepialus armoricanus Oberth) belonging to the family Hepialidae. Adherence of either ascospores or conidia of the fungus with the cuticle of the larva is very common. The fungus colonizes the caterpillars and multiplies inside the host, filling the entire caterpillar with thread like hyphae. The fungus Ophiocordyceps spp are known to produce several secondary metabolites including a nucleoside antibiotic, cordycepin (Isaka et al., 2000) [11]. Ophiocordyceps sinensis is well known for its invigorating and immunological effects on human body (Jiang et al., 2002) [12]. Medicinal properties of O. sinensis are attributed to cordycepin, cordycepic acid, triterpenoids and other active compounds (Rana, 2004) [22]. Investigations have proved that, O. sinensis had possessed anti tumor, free radical scavenging and anticancer principles. It was further suggested that, polysaccharide and sterol fractions of the fungus were responsible for such activities (Zhang et al., 2004 and Wang et al., 2005) [31, 28]. Analytical studies proved that low-molecular weight fractions had showed antimicrobial effects (Kuo et al., 1996) [17]. A number of bioactive compounds from Ophiocordyceps spp have been reported to possess multiple benefits including anti-tumor, anti-microbial, anti-inflammatory and immunomodulatory activities (Schuller and Anke, 2009) [24]. Ophiocordyceps spp extract has been known to contain cordycepin, cordycepic acid, N-acetylgalactosamine, adenosine, ergosterol, β-glucans, exo-polysaccharides and cordymin (Cunningham et al., 1950; Holliday and Cleaver, 2008; Yan et al., 2010 and Wonga et al., 2011) [8, 7, 29, 30]. Among these molecules cordycepin (3’-deoxyadenosine) was the first compound isolated from C. militaris (Cunningham et al., 1950) [3]. Bentley et al. (1951) [21] reported that the cordycepin would be an adenine nucleoside containing a 3’-deoxypentose with a branched carbon chain. The structure of cordycepin is very much similar with cellular nucleoside(adenosine),but lacks 3’ hydroxyl group facilitating the compound to act as anucleoside antibiotic. The cordycepin is known to have many biological activities including the inhibition of cell proliferation, platelet aggregation, cell migration and inflammation (Kim et al., 2006 and Cho et al., 2007) [15, 3]. Apparently, a large part of the metabolism of C. militaris is directed towards the production of cordycepin (25-100 mg per L of medium). But, the biosynthesis depends upon the method of isolation, which may require around 20 days of incubation (Phillips, 1960) [21].
Hsu et al. (2002) [8] indicated that the content of 3′-deoxyadenosine in the mycelial fermentation process would be more (40.8 mg /g) as compared to recovery from fruiting bodies (less than 5.4 mg /g). Therefore, the present investigation conducted for the isolation of most important pharmaceutical molecule of cordycepin from the culture filtrates as well as from the mycelium of Ophiocordyceps sinensis and the native isolates of Ophiocordyceps neovolkiana. Further, it was confirmed through the studies of HPLC and NMR.

Materials and Methods

Ophiocordyceps sinensis isolate no.1220 was obtained from Forest Research Institute (FRI), Dehradun, India and a local isolate of Ophiocordyceps sp. was collected from the cadavers of coconut root grub, Leucoholis coneophora Burm at College of Agriculture, Padannakkad (Latitude 12°26′55″N, Longitude 75°11′41″E) Kasaragod, Kerala during March 2014. Morphological identification was done by visual and microscopic examination. The specimens were stored at 4 °C prior to the isolation and purification of the fungal culture. The purified cultures were further identified by molecular techniques.

For the purpose of tissue isolation, the fruiting bodies of wild isolate were cut into small pieces, surface sterilized with one per cent (w/v) sodium hypochlorite for 60 sec. The tissue bits were washed twice with sterile distilled water for 60 sec and placed in sterile Petri plates containing 20 mL of potato dextrose agar. The plates were incubated at 25 °C for 15 days.

Extraction of cordycepin from culture filtrate

Four mycelial discs measuring 6 mm diameter each, cut from the margin of a 5days old colony of O. sinensis and O. neovolkiana were inoculated separately in 250 mL conical flasks containing 100 mL of sterilized mushroom complete (MC) broth in each flask (adjusted to pH 5.5). The flasks were placed on a rotary shaker maintained at 120 rpm and incubated at 25 °C in diffused day light (600-800 lux) for 15 days. After incubation, the culture filtrate and the mycelial mat were separated by filtration through Whatman No.40 filter paper. The filtrate was further centrifuged at 10,000 rpm and the cell free culture (CFC) filtrate was extracted with methanol. Liquid-liquid extraction was carried out three to four times. Each fungal extract of methanolic solvent was evaporated separately under reduced pressure using a rotary evaporator to obtain the residues. The residue was dried and dissolved in methanol (1mg/mL) and filtered with membrane filter (0.2 μm), stored at 4 °C for further studies.

Extraction of cordycepin from the mycelia

Five gram of freeze dried mycelia obtained from the submerged cultures of O. sinensis and O. neovolkiana maintained at 25 °C for 15 days in MC broth (pH 5.5), was powdered separately with liquid nitrogen and extracted three times with equal volume of methanol at the rate of one ml each time. The extracted sample taken in the Eppendorf tubes was centrifuged at 10,000 rpm for 15 min in a bench centrifuge (at 4 °C) and the supernatants were evaporated to dryness, dissolved in methanol and stored at 4 °C for further studies.

Detection of Bioactive Molecules by Thin Layer Chromatography (TLC)

The cordycepin extract from culture filtrate and mycelial mat of O. sinensis and O. neovolkiana were spotted separately on to silica gel 60 TLC plates (60 F 254, 0.12 mm thick, 5×20 cm, Merck, Germany) at the rate of 5 µL /spot along with standard cordycepin was purchased from Sigma- Aldrich, Inc., USA (Product Number C 3394). After drying, Chromatographs were developed using solvent system butanol: water (86:14). The developed TLC plates were air dried overnight to remove the remaining solvents. TLC plates were viewed under UV light at 250 nm. The retention factor (Rf) values of various compounds resolved on TLC plates were calculated using the formula given by Sadasivam and Manickam (1992) [23].

Detection of cordycepin through HPLC

The extracted cordycepin was further confirmed by High Performance Liquid Chromatography (HPLC). The instrumental parameters and details for HPLC are presented below:

<table>
<thead>
<tr>
<th>Instrumental parameters</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name of equipment</td>
<td>Agilent 1200 HPLC system and AB Sciex API 4000</td>
</tr>
<tr>
<td>Column</td>
<td>Atlantis -dC18 (100mm x 2.0 mm,5µm)</td>
</tr>
<tr>
<td>Guard column</td>
<td>C18 (10mm x 2.1 mm, 5 µm)</td>
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<tr>
<td>Detector</td>
<td>Photo-Diode Array (PDA)</td>
</tr>
<tr>
<td>Mobile phase A and B</td>
<td>Methanol and water (20:80)</td>
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<tr>
<td>Injection volume</td>
<td>10 µl</td>
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<tr>
<td>Column temperature</td>
<td>30 °C</td>
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<tr>
<td>Flow rate</td>
<td>1.0 mL /min</td>
</tr>
<tr>
<td>Detection wavelength</td>
<td>254 nm</td>
</tr>
</tbody>
</table>

Cordycepin standard was separately prepared for HPLC, dissolved in methanol and made to 1000 ppm (one mg /mL) and diluted serially with methanol to get 0.5, 5 and 10 ppm. These dilutions were injected in to HPLC column at the rate of 10 µL. The retention time and peak area were recorded. The similarity between cordycepin standard and extracted samples was analyzed by comparing the data.

Proton Nuclear Magnetic Resonance (NMR) Spectrum of cordycepin

The bioactive compound present in CFC filtrate and mycelial mat of O. sinensis and O. neovolkiana was purified by TLC to obtained 50 mg for each sample. The TLC purified bioactive compounds were sent for NMR analysis at the Sophisticated...
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Analytical Instrument Facility (SAIF), Central Drug Research Institute (CSIR), Lucknow.

Results

Purification of cordycepin by TLC

The methanolic fraction of culture filtrate condensate and mycelial mat extract collected on 15 days after inoculation and it was purified by stepwise gradient with butanol: water (86:14) and three fractions were obtained (F1-F3). Presence of cordycepin was found in fraction F2 (R\textsubscript{f} value 0.63). This was sub-fractionated into four fractions (S1-S4) using elution gradient with butanol: water (86:14). Sub fraction S3 was found to carry cordycepin, which was further vacuum dried and crystallized in methanol, which resulted in a creamy white powdery product. Spectral analysis of purified cordycepin was performed using HPLC and proton NMR.

Detection of cordycepin through HPLC

High performance liquid chromatography analysis for the standard (cordycepin) at 254nm recorded a retention time of 5.92 min (Fig 1). Regression analysis data showed a linear response at three different concentrations (0.1, 0.5 and 1.0 ppm). Similarly, cordycepin was detected in the culture filtrate condensate and mycelial mat extract of \textit{O. sinensis} at 254nm with a retention time of 5.84 (Fig 2) at a retention time of 5.9 min (Fig 3). But, \textit{O. neovolkiana} did not show any peak at the same retention time.

H NMR spectrum of bioactive molecules of \textit{O. sinensis}

The proton NMR spectral analysis of cordycepin produced by \textit{O. sinensis} and \textit{O. neovolkiana} (CFC filtrate condensate and mycelial mat extract) showed anomeric carbon peaks at 3.4 ppm (Fig 4, 5, 6 and 7) which was comparable with \textsuperscript{1}H NMR spectrum of standard cordycepin (Fig 8).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{HPLC analysis of cordycepin standard (0.1ppm)}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Culture filtrate condensate contained cordycepin produced by \textit{O. sinensis}}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{Mycelial mat extract contained cordycepin produced by \textit{O. sinensis}}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{H NMR spectral analysis of culture filtrate condensate of \textit{O. sinensis}}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{H NMR spectral analysis of mycelial mat extract of \textit{O. sinensis}}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6.png}
\caption{H NMR spectral analysis of culture filtrates of \textit{O. neovolkiana}}
\end{figure}
Later, Ling militaris D As early in 1950, cordycepin was first isolated from mycelia of and LCMS. In the present study, cordycepin present in was noted, which ranged between 5.84-5.95 min. By HPLC port at the same volume. Here again the retention time extracted metabolite from was found to be 5.9 min. Later, the concentrations extracted metabolite from O. sinensis was purchased from Sigma Aldrich, Inc., best solvent for dissolving the compound. In addition, the solvent system used for extraction was butanol: water (86:14). Further, it was revealed that, methanol would be the best solvent for dissolving the compound. In addition, the proton NMR spectral analysis of cordycepin purified from the CFC filtrate condensate and mycelial mat extract of O. sinensis and O. neovolkiana demonstrated the presence of anomeric carbon peaks at 3.4 ppm (Fig 4, 5, 6, 7 and 8). This observation has been further compared with 1H NMR spectrum of standard cordycepin reported by Tuli et al. (2014) [23]. Further, the NMR analytical data obtained during the current investigation was found to be consistent with the earlier reports (Cunningham et al., 1950 and Chatterjee et al., 1957) [5, 2].

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References