

Effect of Cordycepin Purified from *Cordyceps militaris* on Th1 and Th2 Cytokines in Mouse Splenocytes

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Cordycepin was purified from a mushroom, *Cordyceps militaris*, and its effect on Th1 and Th2 cytokines was examined. The level of cytokine induction in mouse splenocytes was estimated after co-inoculation of purified cordycepin and LPS. When 5 µg/ml of purified cordycepin was exposed to mouse splenocytes for 72 h, the level of a Th1 cytokine IL-12 increased by 2.9-fold. The addition of the purified cordycepin to splenocytes also increased the level of Th2 cytokines, IL-4 and IL-10, by 1.9- and 1.8-fold, respectively. Therefore, cordycepin increases the cytokine levels and may contribute to the up-regulation of cellular and humoral immunity.

Keywords: Cordycepin, *Cordyceps militaris*, IL-4, IL-10, IL-12

Cordyceps militaris is a traditional medicinal mushroom that has been shown to have a variety of benefits on human health, such as antitumor [18], antimutagenic [3], and hypoglycemic effects [4]. Cordycepin is involved in many pharmacological activities, including immunological stimulating, anticancer, antiviral, and anti-infection activities [10, 17]. A variety of chemical constituents, including cordycepin, polysaccharides, and glycoproteins, are involved in these

activities [2]. A nucleoside analog (3'-deoxyadenosine), cordycepin, which was first reported as a metabolite isolated from *C. militaris*, is one of the major active constituents [5]. Cordycepin has been shown to display antitumor, antibacterial, antiviral, immunomodulatory, and anti-inflammatory activities [8, 9, 11, 18].

Cytokines are signaling molecules involved in host defense, growth, and repair processes within injured tissues [7]. There are Th1 and Th2 cells in T helper lymphocytes and each cell type secretes different types of cytokines. It is known that Th1 cell subsets secrete interferon (IFN)- γ , tumor necrosis factor (TNF)- α/β , interleukin (IL)-2, and IL-10, and Th2 cell subsets secrete IL-4 and IL-10. IL-4 induces differentiation of naive helper T (Th0) cells to Th2 cells. It performs as a regulator in humoral and adaptive immunity. Human cytokine synthesis inhibitory factor (CSIF) IL-10 is an anti-inflammatory cytokine. IL-12 is also involved in the development of Th0 cells into either Th1 cells or Th2 cells [1, 13–16].

However, the effect of cordycepin purified from *C. militaris* on Th1 and Th2 cells cytokines secreted from mouse splenocytes has not yet been elucidated. In this paper, we investigated the effect of purified *C. militaris* cordycepin on cytokines secreted by T helper lymphocytes in lipopolysaccharide (LPS)-stimulated mouse splenocytes.

C. militaris used in this work was obtained from a Cheongweon-Industrial farm (Gimhae, Korea), where the mushroom was cultured and lyophilized. Various strains of *C. militaris* were constructed by single spore fusion, producing a strain, *C. militaris* JLM 0636. The strain was stored on a potato dextrose agar (PDA) (Difco, Franklin

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Lakes, USA) slant. Prior to the experiment, a piece of mycelium in the stock culture of the strain was transferred to a fresh PDA slant and incubated at 25°C for 14 days. The inoculum was prepared by punching out a 1 cm disc from the PDA plate with a sterilized cork borer. The microbial strain was cultured by inoculating a seed disc into 500 ml Erlenmeyer flasks.

A hot water extract of the *C. militaris* strain was prepared by autoclaving the culture at 121°C for 3 h, cooling to room temperature, filtering through a 0.45 µm membrane, and removing the suspended mycelia. Extraction and purification of cordycepin from *C. militaris* were performed using the He Ni method [12]. HPLC was performed using a Perkin-Elmer Series 200, equipped with UV detector (260 nm) and Bondapak C18 column (19 × 300 mm, 20 mm; Waters Co., Baltimore, USA). The mobile phase was water:MeOH = 9:1 (v/v) and the flow rate was 5 ml/min. Analytical HPLC was performed using a Bridge C18 column (4.6 × 250 mm, 3.5 mm; Waters Co., USA). The mobile phase consisted of the following linear gradient: A, 0.01 M Phosphate buffer (pH 6.5)/B, MeOH; 0–15% B for 10 min, 15–10% B for 5 min, 10–20% B for 2 min, and 20% B for 12 min. The column oven was kept at 40°C and the injection volume was 10 µl.

For experimental animals, C57BL6 mice (6–8 weeks old) were purchased from the DBL company (Umsung, Korea) and housed in a specific pathogen-free animal facility for at least one week before use. All procedures involving animals were approved by the Animal Care Committee of Kosin Medical School, Busan, Korea. Splenocytes were isolated from each animal by aseptically removing the spleens from the mice. Mouse spleens were homogenized in a tissue culture medium (Celox Laboratories Inc., Lake Zurich, USA), and cultured in a RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml of penicillin, 100 µg/ml of streptomycin, 0.25 µg/ml of amphotericin B, and 10% (v/v) heat-inactivated fetal bovine serum. Mouse splenocytes (1 ml/well), in the absence or presence of LPS (2 µg/ml), were cultured in 24-well plates and incubated at 37°C in a humidified incubator supplied with 5% CO₂ and 95% air for 0, 24, 48, and 72 h. The cultured cells were discarded by centrifuging at 1,500 ×g for 10 min. Then the supernatants were collected and stored at –70°C until analyzed. The levels of IL-2, IL-4, IL-10, and IL-12 were measured using the Max set (BioLegend, San Diego, USA), according to the manufacturer's instructions. Briefly, a 96-well flat-bottom plate was coated with capture antibody specific to each cytokine. The plate was washed and blocked before 100 µl of the supernatants was added, and serially diluted specific standards were added to the respective wells. Following a series of washing, the captured cytokine was detected using the specific biotinylated detection antibody. The avidin-HRP/TMB substrate reagent was added to each

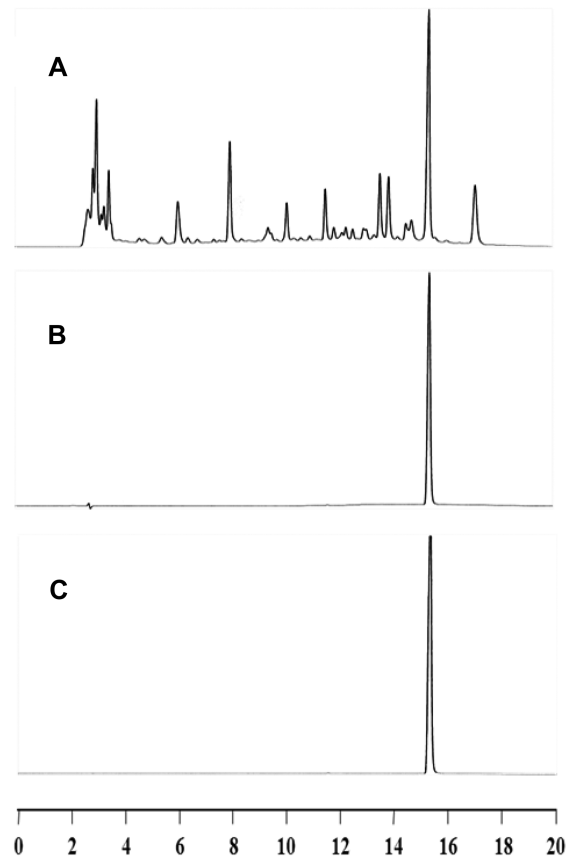


Fig. 1. Identification of cordycepin.

(A) Hot water extract of *C. militaris* JLM 0636. (B) Purified cordycepin. (C) Commercial cordycepin.

well and, after color development, the plate was read at 450 nm, using an ELISA plate reader (Bio-Rad, Hercules, USA).

During the process of cordycepin purification, 4.6 mg of cordycepin was produced from 1 g of the *C. militaris* extract on a dry weight. The HPLC spectra of both the hot water extract of *C. militaris* and cordycepin purified from the mushroom were compared. For purification of cordycepin, the hot water extract of the strain was firstly prepared and subjected to HPLC analysis. The resulting HPLC spectrum contained many peaks (Fig. 1A). The purity of cordycepin prepared from the hot water extract was approximately 93% (Fig. 1B), which was determined, relative to commercial cordycepin (Sigma, St. Louis, USA) that had a purity of 95% (Fig. 1C). Results from the analysis of the spectra showed that the effective component isolated from *C. militaris* was nearly identical to commercial cordycepin. To examine the anti-inflammatory activity of cordycepin on mouse splenocytes, both 5 µg/ml of purified cordycepin and LPS were co-administered to mouse splenocytes for 0, 24, 48, and 72 h. The level of Th1 cytokine IL-12 in the cells exposed to the purified

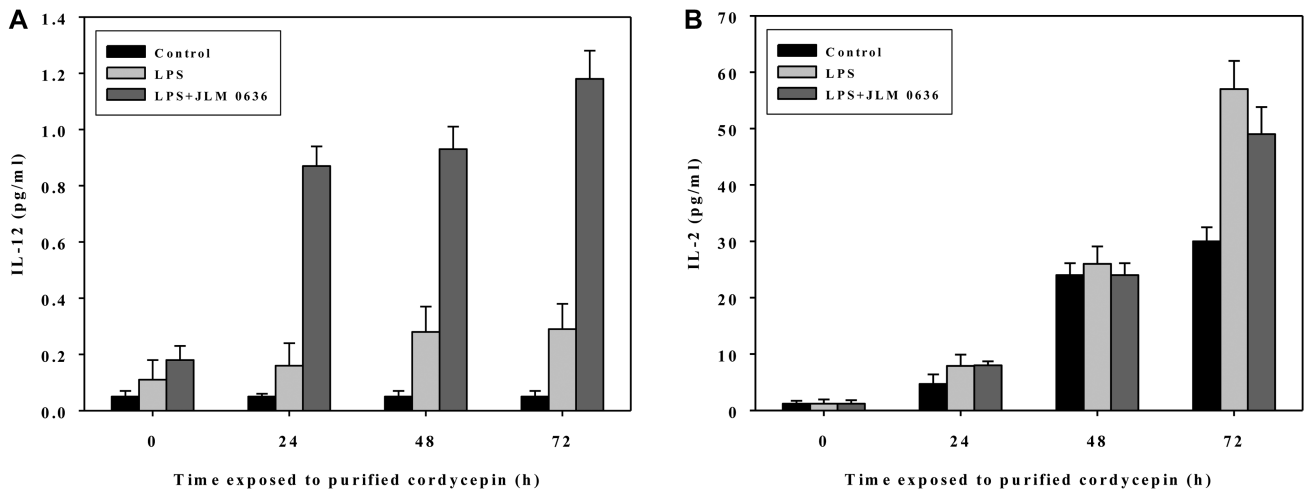


Fig. 2. Effect of cordycepin purified from *C. militaris* on Th1 cytokines IL-12 (A) and IL-2 (B) in cultured LPS-treated mouse splenocytes.

Five $\mu\text{g/ml}$ of purified cordycepin was exposed to mouse splenocytes. Data are expressed as the mean \pm SD of three independent experiments performed in duplicate.

cordycepin and LPS for 72 h was 2.9-fold, higher than cells treated with LPS only (Fig. 2A); however, the simultaneous addition of *C. militaris* cordycepin and LPS did not result in significant IL-2 induction (Fig. 2B). In addition, a significant increase in IL-2 levels was observed when the cells were treated with both purified cordycepin and LPS for 24 h. The amounts of Th2 cytokines IL-4 and IL-10 levels in mouse splenocytes were also estimated (Fig. 3). Compared with LPS-treated cells, exposure to the same concentration of the purified cordycepin and LPS increased the IL-4 and IL-10 levels by 1.9- and 1.8-fold, respectively. Therefore, these findings may indicate that

cordycepin purified from the mushroom exerts both cellular and humoral immunity in mouse splenocytes by increasing the levels of several cytokines, including IL-4, IL-10, and IL-12. The potential of cordycepin to favor Th1 and Th2 immune activities may be beneficial for treating both Th1-skewed diseases, including type 1 diabetes, multiple sclerosis, Crohn's disease, and rheumatoid arthritis, and Th2-skewed diseases such as allergies, systemic lupus erythematosus, and progressive systemic sclerosis [6]. Therefore, cordycepin could be used in formulations for treating diseases or to develop functional foods with immunomodulatory properties.

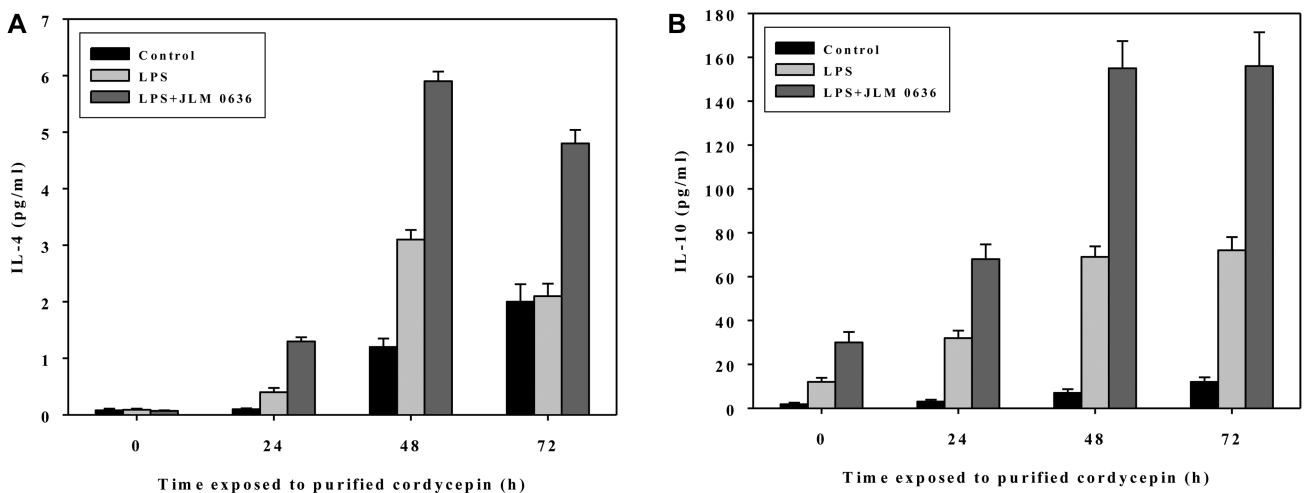


Fig. 3. Effect of cordycepin purified from *C. militaris* on Th2 cytokines IL-4 (A) and IL-10 (B) in cultured LPS-treated mouse splenocytes.

Five $\mu\text{g/ml}$ of purified cordycepin was exposed to mouse splenocytes. Data are expressed as the mean \pm SD of three independent experiments performed in duplicate.

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