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Macrophage biospecific extraction and high performance liquid chromatography for hypothesis of immunological active components in *Cordyceps sinensis*

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Abstract

A method, namely macrophage biospecific extraction and high performance liquid chromatography for screening potential immunological active components in *Cordyceps sinensis*, a well-known traditional Chinese medicine, was developed. Two components, which could interact with macrophage, in aqueous extract of *C. sinensis* (WECS) were found by comparing the HPLC chromatograms of WECS before and after interacted with macrophage. The two compounds were identified as guanosine and adenosine. Their effects on mice macrophage were also investigated *in vitro*. The results showed that adenosine and guanosine could attenuate NO ($p < 0.01$) but augment interleukin-lβ (IL-1β) ($p < 0.05$) release of macrophage during the tested concentrations. In addition, guanosine (0.10 μmol/ml) also increased alpha-tumor necrosis factor (TNF-α) release of macrophage. The data suggest that macrophage biospecific extraction and HPLC is a useful method to screen immunological active components from Chinese medicines.

Keywords: Macrophage biospecific extraction; HPLC; *Cordyceps sinensis*; Immunological active component

1. Introduction

Cordyceps sinensis, one of the most valued traditional Chinese medicines (TCMs), consists of the dried fungus *C. sinensis* growing on the larva of caterpillar. It is also known as "summer-grass and winter worm" because of its appearance during the different seasons. The parasitic complex of the fungus and the caterpillar is found in the soil of a prairie at an elevation of 3500–5000 m. It is commonly used in China to replenish the kidney and soothe the lung for the treatment of multiple diseases [1,2]. The fruiting body (fungus) and the worm (caterpillar) show very similar profiles of chemical compositions and also pharmacological properties [3], which suggest the total invasion of the caterpillar by the *Cordyceps* mycelia. Several mycelial strains have been isolated from natural *Cordyceps* and produced in large quantity by fermentation. These artificial products possess similar pharmacological effects to the natural counterparts [1,2,4]. Recent studies have demonstrated the multiple pharmacological actions, such as the effects on the immune system, of *Cordyceps* [5–7]. However, the experimental results are rather controversial that *Cordyceps* possesses both potentiation and/or inhibition effects on immune response [8–11]. Thus, it is assumed that *Cordyceps* is a bi-directional modulator of immune system. On the other hand, immunological active compounds in *C. sinensis* are not clearly known. Therefore, the screening and analysis of bioactive components in *C. sinensis* is very important not only for quality control but also for elucidation of the therapeutic mechanism. Modern pharmacological studies have shown that combination with some receptors or channels on cell membrane is the first step of drug action. Therefore, the ability of a drug to interact with cell membrane is very important for the behavior of the drug in the organism. In

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our previous study, four potential active candidates in *Angelica sinensis* were hypothesized by using human red cell membrane extraction and high performance liquid chromatography [12].

In this paper, for screening potential immunological active components in *C. sinensis*, macrophage, a phagocyte cell that helps initiate and is involved in all stages of immune responses, was used for biospecific extraction of aqueous extract of *C. sinensis*, and then high performance liquid chromatography was performed. Two potential active candidate compounds, which could interact with macrophage, in *C. sinensis* were discovered. Their effects on cytokines release of macrophage were also investigated *in vitro*.

2. Experimental

2.1. Materials

Cultured *C. sinensis* was obtained from Jiangxi, China. The species identity of this cultured *Cordyceps* was guaranteed by State Food and Drug Administration of China. Nucleosides such as adenosine, cytidine, guanosine, thymidine, uridine and their bases cytosine, guanine, thymine and uracil, as well as disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride were purchased from Sigma (St. Louis, USA). Methanol for LC, acetic acid and concentrated Giemsa stain were purchased from Merck (Darmstadt, Germany). RPMI 1640 media were from GIBCO (Paisley, Scotland, UK). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA).

2.2. Extraction of chemical constitutes from C. sinensis

About 100 g of cultured *Cordyceps* powder was soaked in 600 ml water and then boiled for 1 h. After centrifugation at 3000 rpm for 10 min, the supernatant was collected and lyophilized. Then the extract was dissolved in 1000 ml of 5 mM phosphate buffer (pH 7.4) and the chromatographic characteristics for water extract of *C. sinensis* (WECS) was determined using HPLC.

2.3. Preparation of macrophages

The experiments were conducted in primary cultures of macrophages obtained from pathogen-free adult Wistar rats of both sexes, weighing approximately 210 g each. All animal were maintained on an ad libitum diet of laboratory chow and water. The University Animal Care Committee approved all protocols. A ventral incision was made, carrier fluid was added, and the abdomen was massaged manually. The carrier fluid gently was transferred by pipette into a sterile polypropylene spin tube. Carrier fluid was incubated in collagen-coated dishes for 3 h at 37 °C in a humidified atmosphere (95% air and 5% CO_2), the nonadhesive cells were removed from the wells by three washes of prewarmed Dulbecco's phosphate-buffered saline (DPBS), and the remaining adhesive cells were suspended with phenol red-free RPMI 1640 medium to obtain a protein concentration of 10 g/l as measured by absorbance at 280 nm [13] and finally the prepared material was used for macrophage biospecific extraction. More than 96% of the adhesive cells were nonspecific esterase-positive and had the morphological appearance of macrophages examined after Giemsa staining.

2.4. Macrophage biospecific extraction of sample

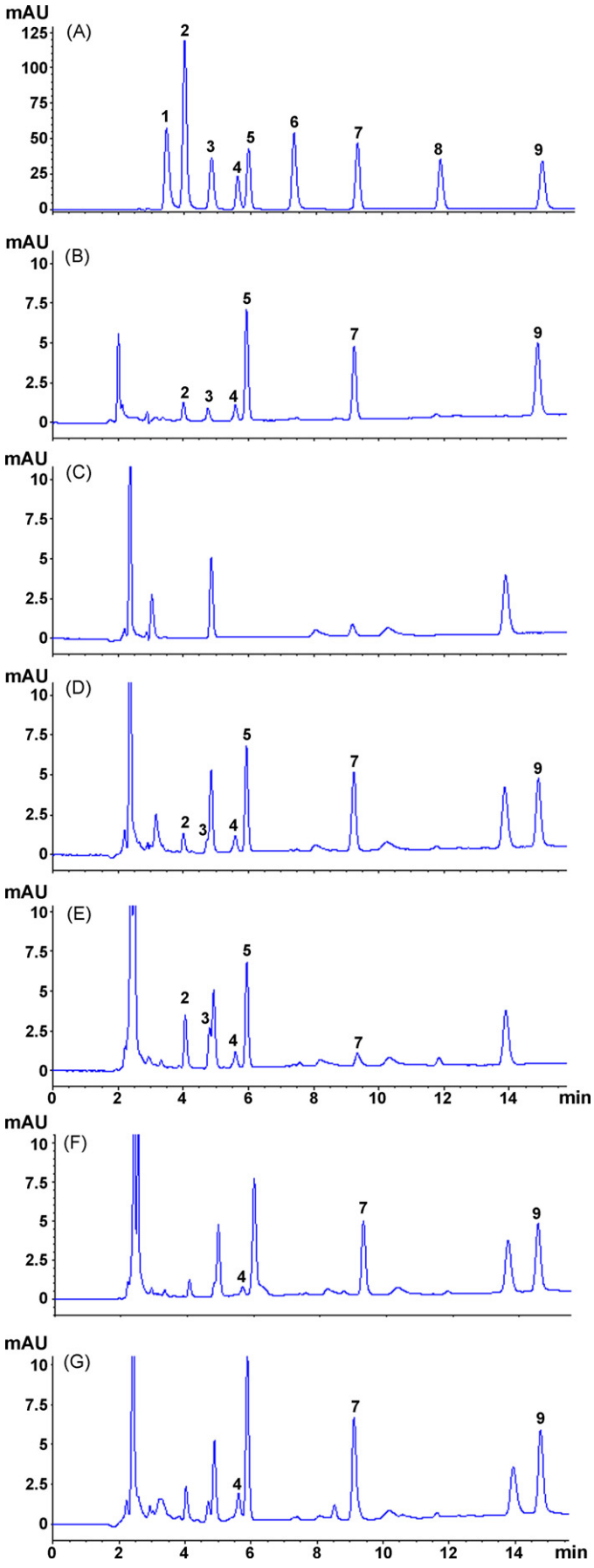
The same volume of macrophage suspension and 5 ml WECS were incubated with shaking for 30 min at 37 °C. The suspension was then transferred to Centriprep YM-50 centrifugal filters and centrifuged at 1000 × *g* for 10 min or until the filter appears dry. The filtrate was obtained for HPLC analysis. The cells collected on the filter were washed three times to remove the unbound components. Each wash was accomplished by addition of 1 ml of RPMI 1640 medium to the filter with subsequent centrifugation at 1000 × *g* for 10 min or until dry. The washing eluate was discarded except the last one which was collected as control for HPLC analysis. Finally, macrophages were denatured by addition of 1 ml of 20% acetic acid in water to the filter, and the bound components were delivered. The released components were collected by centrifugation into a fresh collection vessel. The desorption eluate from this step is evaporated to dryness by centrifugation in vacuo (SpeedVac, Savant) and reconstituted by sonication in 0.2 ml of a 1:1 acetonitrile/16 mM aqueous ammonium acetate solution immediately before analysis. The blank desorption eluate, replaced WECS with RPMI 1640 medium to interact with macrophages, was obtained using the method described above, and HPLC analysis of the blank was performed.

2.5. HPLC analysis

Analysis were performed on an Agilent Series 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA), equipped with a vacuum degasser, a quaternary pump, an autosampler and a DAD detector, connected to an Agilent ChemStation software. A Zorbax 300SB C18 column (4.6 mm × 250 mm i.d., 5 μm) and a Zorbax ODS C18 guard column (4.6 mm × 12.5 mm i.d., 5 μm) were used. HPLC–MS was performed as our reported method [14] with minor modification. Solvents that constituted the mobile phase were: (A) 8 mM aqueous ammonium acetate and (B) methanol. The elution conditions applied were: 0–20 min, linear gradient 5–25% B and finally, reconditioning steps of the column was 5% B isocratic for 15 min. The flow-rate was 1 ml/min and the injection volume was 20 μl. The system was operated at 25 °C. Peaks were detected at 260 nm.

2.6. Mass spectrometry

The HPLC conditions for LC–MS analyses were the same as those used for HPLC analysis described in Section 2.5. Agilent 1100 Series LC/MSD Trap (Agilent Technologies) ion-trap mass spectrometer with electrospray ionization interface, connected to an Agilent ChemStation software was used in HPLC–MS method. ESI-MS conditions were as follows: positive ion mode,



drying gas N_2, 10 l/min, temperature 350 °C, pressure of nebulizer 40 psi and caplillary voltage 4.5 kV, scan range 50–500 u. ESI-MS/MS conditions were as follows: positive ion mode, separation width 0.9, fragment amplification 1.

2.7. Assay for cytokines release of macrophage

The effects of two compounds, guanosine and adenosine, on cytokines release of macrophage from mice (20–24 g) were tested. Macrophages obtained as mentioned above were adjusted to 2.0–2.5 × 10^6 cells/ml. The cells were then added in each well of 24-well plates in 900 μl medium and treated as follows: (1) control, adding 100 μl of normal saline; (2) adenosine, adding 100 μl adenosine with concentration of 3.0 or 15.0 μmol/ml; (3) guanosine, adding 100 μl guanosine with concentration of 0.2 or 1.0 μmol/ml. The cells were cultured in RPMI 1640 with 10% fetal bovine serum at 37 °C at a humidified 5% CO_2 atmosphere for 24 h. One-hundred microliter medium of each well was used to detect the level of nitric oxide (NO), alpha-tumor necrosis factor (TNF-α) and interleukin-lβ (IL-1β). The level of NO was measured indirectly by determining the level of nitrite in cell culture supernatants using the Griess assay [15]. Enzymelinked immunosorbent assay (ELISA) kits (Jingmei Biotech Co., Ltd. Nanjing, China) were used for determination of TNF-α and IL-1β.

2.8. Statistical analysis

Statistical analysis was performed using Student's *t*-test for significance and a *p*-value (two-tailed) less than 0.05 was regarded as significant.

3. Results and discussion

3.1. Macrophage biospecific extraction of compounds in C. sinensis

Affinity methods is unique among separation methods as it is the only technique that permits the purification of compounds based on biological functions rather than individual physical or chemical properties [16]. Compounds with affinities similar to

Fig. 1. HPLC chromatograms of: (A) mixed reference nucleosides, (B) aqueous extract of *Cordyceps sinensis* (WECS), (C) RPMI 1640 solution, (D) WECS with RPMI 1640 solution, (E) supernatant of WECS after incubation with macrophage, (F) supernatant of WECS after incubation with heat denatured macrophage, and (G) supernatant of WECS after incubation with human hepatoma SMMC-7721 cell. HPLC analysis were performed on an Agilent Series 1100 liquid chromatograph, equipped with a vacuum degasser, a quaternary pump, an autosampler and a DAD detector, connected to an Agilent ChemStation software. A Zorbax 300SB C18 column (4.6 mm × 250 mm i.d., 5 μm) and a Zorbax ODS C18 guard column (4.6 mm × 12.5 mm i.d., 5 μm) were used. Solvents that constituted the mobile phase were (A) 8 mM aqueous ammonium acetate and (B) methanol. The elution conditions applied were: 0–20 min, linear gradient 5–25% B and finally, reconditioning steps of the column was 5% B isocratic for 15 min. The flow-rate was 1 ml/min and the injection volume was 20 μl. The system operated at 25 °C. Peaks were detected at 260 nm: 1, cytosine; 2, uracil; 3, cytidine; 4, guanine; 5, uridine; 6, thymine; 7, guanosine; 8, thymidine; 9, adenosine.

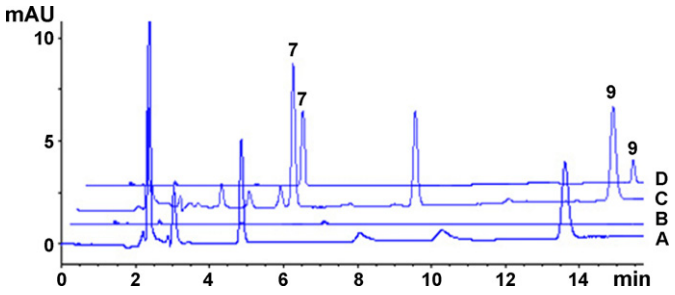


Fig. 2. HPLC chromatograms of: (A) RPMI 1640 final washing eluate of WECS interacted with macrophage, (B) desorption eluate of phosphate buffer interacted with macrophage, (C) aqueous extract of *Cordyceps sinensis* (WECS), and (D) desorption eluate of WECS interacted with macrophage. HPLC condition was the same as that described in Fig. 1. Peak 7, guanosine; peak 9, adenosine.

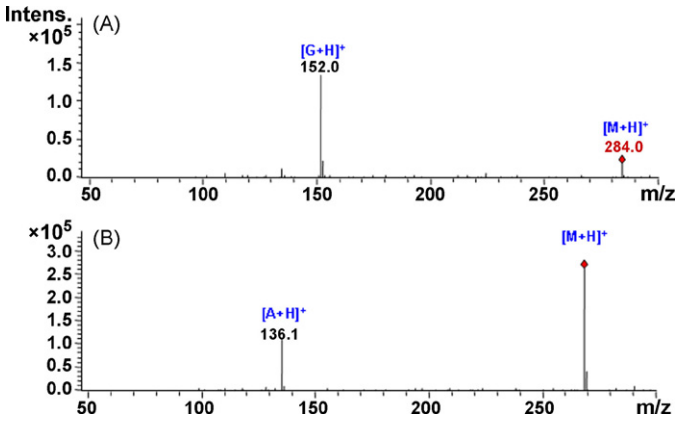


Fig. 3. MS data of: (A) peak 7 and (B) peak 9 on HPLC profile of WECS incubated with macrophage. HPLC condition was the same as that described in Fig. 1. Peak 7, guanosine; peak 9, adnenosine. A, adenine; G, guanine.

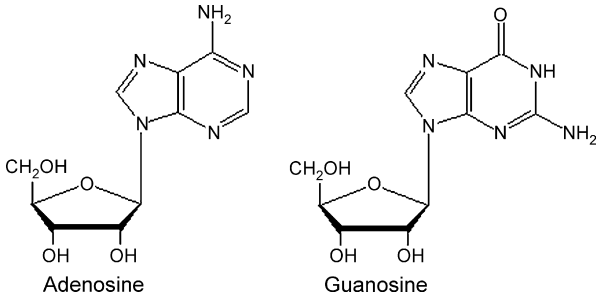


Fig. 4. Structures of guanosine and adenosine.

a target receptor are also likely to share the same pharmacological properties. An optimal method for screening of potential active components in TCMs using biomembrane extraction and HPLC analysis was reported [12]. In our paper, macrophage was chosen for screening immune modulators in *C. sinensis*. The components were monitored at 260 nm for HPLC analysis. HPLC chromatograms of WECS before and after biospecific extraction with macrophage under the experimental conditions described above were shown in Fig. 1. There are two peaks, i.e. peak 7 and 9, on chromatogram of WECS significantly decreased or even disappeared after interaction with macrophages. However, the chromatograms of WECS were same before and after incubating with heat denatured macrophage (Fig. 1F) or human hepatoma SMMC-7721 cell (Fig. 1G). The results suggested that the two components could specifically interact with macrophage (Fig. 2).

MS is a powerful technique for identification of molecular structure. However, the fractions of peaks separated by immobilized biomembrane affinity chromatography could not be directly applied for MS analysis because of the presence of a high concentration of inorganic salts [17]. Using LC-DAD-MS/MS analysis, the components of peaks on chromatogram of WECS extract could be directly identified. By comparison of their retention time and their UV spectra with those obtained injecting nucleosides standards in the same conditions and their MS data (Fig. 3), the components for peak 7 and 9 were identified as guanosine and adenosine, respectively (Fig. 4).

3.2. Effects of two identified compounds on cytokines release of macrophage

The effects of the two identified compounds, adenosine and guanosine, on cytokines release of macrophage were shown in Table 1. The results showed that both could attenuate NO ($p<0.01$) but augment IL-1β ($p<0.05$) release of macrophage during the tested concentrations. In addition, guanosine (0.10 μmol/ml) also increased TNF-α release of macrophage, but adenosine seemed had various effects on release of TNF-α from macrophage at different concentrations though it was not significant. The results suggested that adenosine and guanosine may have multiple effects on macrophage.

The activation of macrophages plays a key role in innate immunity, which participates actively in host defense against

Table 1
Release of NO, TNF-α and IL-1β in mice macrophage treated with two identified compounds, adenosine and guanosine, for 24 h ($n=3$)

Compounds	Concentration (μmol/ml)	NO (μmol/l)	TNF-α (pg/ml)	IL-1β (pg/ml)
RPMI 1640	–	30.62 ± 1.33	10.80 ± 1.48	44.01 ± 12.74
Adenosine	0.30	28.10 ± 0.77*	13.04 ± 8.86	104.84 ± 32.58**
	1.50	25.90 ± 0.50**	7.54 ± 2.80	410.58 ± 105.95**
Guanosine	0.02	28.93 ± 1.03	17.75 ± 5.32	32.32 ± 4.92
	0.10	27.46 ± 0.42**	23.55 ± 1.34**	177.85 ± 79.57**

* $p<0.05$.
** $p<0.01$ vs. control (RPMI 1640).

pathogen infection, through phagocytosis and pathogen killing, and in inflammatory reactions. Elevated nitric oxide synthesis after the induced expression of NOS-2 by activated macrophages is one of the main cytostatic, cytotoxic, and pro-apoptotic mechanisms participating in the innate response in many mammals [18]. Adenosine decrease the level of NO may derived from its inhibition on macrophage. In addition, alpha-tumor necrosis factor is a potent proinflammatory cytokine and a critical mediator of inflammatory responses in mammals, and it also play prominent roles in bridging the innate and adaptive phases of immunity [19]. TNF-α is cytotoxic to lymphocytes and in some situations can act to suppress the immune system. Guanosine may modulate the immune responses through influence the activation of macrophage. Actually, TNF-α and IL-1β are regulated by similar promoter elements and have been shown alike response to stimuli. Therefore, adenosine and guanosine may increase the secretion of TNF-α and IL-1β from macrophage through the same pathway. The immunological activities of them should be further investigated.

4. Conclusion

Two immunological active components in *C. sinensis* were found using macrophage biospecific extraction and HPLC, which suggested that the method is useful to screen active components from Chinese medicines. In addition, HPLC coupled with other complementary techniques such as MS and NMR will further improve the structural identification of potential active compounds.

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