Effects of heat on the biological activity of wild *Cordyceps sinensis*

Pengkai Wu, Zhi Tao, Huafeng Liu, Guixiang Jiang, Changhua Ma, Chunmei Wang*, Di Geng

Department of Biopharmaceuticals, School of Chinese Materia Medica, Beijing University of Chinese Medicine, Beijing 100102, China

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**Abstract**

**Background:** Current methods of extending the storage time of wild *Cordyceps sinensis* adversely affect the nutritive and medicinal value of the product. Thus, this study was designed to investigate the effects of heat treatment, a relatively safe storage extension method, on the biological activity of wild *C. sinensis*.

**Methods:** Samples were heated to 60, 80, or 100°C for 15, 30, or 60 minutes. SOD activity in wild *C. sinensis* before and after heating was assayed using a standard colorimetric assay. Deoxyribonuclease (DNase) activity was measured using the plasmid-nicking assay. Cordycepin content was analyzed using HPLC. Polysaccharide content was measured using the phenol-sulfuric method. The Student’s *t*-test was used for comparison.

**Results:** After heating at 60, 80, 100°C for 15, 30, 60 minutes, respectively, no significant reduction in DNase activity or polysaccharide dissolution was noted (*P* > .05). Interestingly, heating at 80°C for 30 minutes led to a significant increase in the SOD activity of *C. sinensis* (*P* < .05). In addition, heating at 60°C for 60 minutes or at 80°C for 15 minutes significantly increased cordycepin dissolution (*P* < .05). Other heat treatments had no significant effects on SOD activity or cordycepin dissolution (*P* > .05).

**Conclusions:** These results suggested that heat treatment does not adversely affect SOD activity or cordycepin dissolution. Thus, heat treatment might be a safe processing method to extend the storage time of wild *C. sinensis* without compromising biological activity.

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Introduction

*Cordyceps sinensis*, a fungus belonging to the family Clavicipitaceae, is a rare mushroom well known in China, where it is called Dong Chong Xia Cao (meaning winter worm, summer grass). It is found in China, the Tibetan Plateau, Bhutan, Nepal, and the northeastern regions of India, at altitudes of approximately 3500–5000 meters above sea level. However, wild or natural *C. sinensis* is becoming increasingly scarce because of reckless harvesting, its limited distribution, and weather conditions unfavorable for its proliferation.

Wild *C. sinensis* has been used in traditional Chinese medicine for centuries, mainly as a tonic to invigorate the lungs and nourish the kidneys. It is marketed as a dietary supplement by the FDA (U.S. Food and Drug Administration), and hence market demand for wild *C. sinensis* is high in Asian countries. As a result, wild *C. sinensis* is very expensive, and the trade in wild *C. sinensis* has become very active owing to marked commercial interests. However, the storage of wild *C. sinensis* is difficult because of its rich nutrition. The transportation and storage process renders the plant vulnerable to damage by mildew and insects. Traditionally, wild *C. sinensis* was cleaned after harvesting, dried, and processed with a fumigant to extend storage time. Unfortunately, sulfur-fumigant treatment introduces SO$_2$, which is toxic to human health, into the products. The use of vacuum maintenance has also been attempted in the storage of wild *C. sinensis*, and increased storage time. However, current commercial distribution enterprises do not satisfy with these conditions for the storage of wild *C. sinensis*.

Heat treatment is one of the primary means by which humans change the composition and chemistry of food tissues, making them more digestible, less toxic, and more durable. This technology that has long been used by the food industry might be a safe, simple, and effective means to prolong the storage time of *C. sinensis*. Contamination by microorganisms on the surfaces of wild *C. sinensis* primarily occurs after harvesting; therefore, suitable surface heat treatments could be valuable microbicidal and insecticidal measures.

Thus, this study aimed to observe the effect of heat treatment on the biological activity of wild *C. sinensis* and find an efficient heat treatment method to increase shelf life and reduce economic losses without adversely affecting the medicinal value of *C. sinensis*.

Methods

Reagents

Sulfuric acid (PubChem CID: 1118) 96.8 g/dL, phenol (PubChem CID: 996) 5 g/dL, D-glucose (PubChem CID: 5793), ethanol (PubChem CID: 702), and methanol (PubChem CID: 887) were obtained from Sigma Co. (St. Louis, MO, USA).

Heat treatment of *C. sinensis*

Wild *C. sinensis* specimens were collected from the Qinghai-Tibet plateau (Nagqu County, Biru district, Tibet province), at an altitude of over 4000 meters. Specimens were authenticated by Professor Chang Hua Ma of the School of Chinese Materia Medica, Beijing University of Chinese Medicine. The specimens were cleaned (Fig. 1), and heated at 60, 80, or 100 °C for 15, 30, or 60 minutes using a heat block (Dry Thermo Unit, Yiheng Scientific Instruments, Shanghai, China); thus, 10 treatment groups were used in total. These specific temperatures and time periods were selected considering that breeding bacteria can be killed by treatment at 100 °C for 60 minutes, and most viruses can be killed by treatment at 60 °C for 30 minutes.

Relevant chemical constituents and biological activities of these heat-treated samples were then determined to examine changes in the biological activities of wild *C. sinensis*. *C. sinensis* specimens that had not been exposed to heat were used as negative controls.

Preparation of *C. sinensis* aqueous extracts

One gram of *C. sinensis* was ground using a cold mortar on ice for 10 minutes, and was dissolved in 5 mL distilled water for 30 minutes in 4 °C. Next, it was centrifuged at 4722 g for 10 minutes. The supernatant fluid was stored at −20 °C until use. The resultant aqueous extract is a 1:5 (w/v) extract; the *C. sinensis* homogenate-to-water ratio was chosen following methods used by Song et al.

Determination of cordycepin

The aqueous extracts was filtered through a 0.45 μm membrane and then analyzed by high-performance liquid chromatographic (HPLC) using a Model 1100 series LC system (Agilent Technologies, Palo Alto, CA, USA). Chromatographic separation was performed on a 4.6 mm × 250 mm, 5 μm Zorbax StableBond Analytical SB-C18 column (4.6 mm × 250 mm, 5 μm, Agilent Technologies). The mobile phase was composed of water and methanol (85:15 v/v). The flow rate was 1 mL/min. The UV wavelength was set at 260 nm and the column temperature at 30 °C.
Measurement of superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined using a modified pyrogallol autoxidation procedure. Aqueous extracts of Nagqu C. sinensis were added to Tris buffer at pH 8.2 (final concentration 50 mM, containing 1 mM EDTA). After vortexing for 1 minute, the reaction was initiated by the addition of pyrogallol at a final concentration of 0.2 mM. The change in absorbance at 325 nm was measured every 30 seconds for 4 minutes at 25°C. Results were expressed as units of enzyme activity per gram of sample.

Measurement of DNase activity

DNase activity was monitored using a plasmid-nicking assay, using supercoiled pUC18 as a substrate. C. sinensis aqueous extracts (10 μL) and 2 μg of the substrate plasmid were added to the reaction buffer (500 mM sodium acetate, 5 mM EDTA, pH 5.5) and incubated for 30 minutes at 37°C. Then, the reaction was stopped by the addition of 20 μL of 0.1 M HCl and 80 μL of 95% ethanol was added to the mixture. After being allowed to stand for 5 minutes at 25°C, the mixture was centrifuged at 7378 × g for 30 seconds and the absorbance of the supernatant (diluted 10-fold with sterile water) was measured at 260 nm. One unit of DNase activity was defined as the amount of enzyme that increased absorbance of the supernatant at 260 nm by 1.0 in 10 minutes under the above conditions.

Determination of polysaccharide dissolution

One gram of the C. sinensis was homogenized using a cold mortar and dissolved in 5 mL distilled water. To remove lipids, the sample was added to a beaker containing 50 mL ethanol and then sonicated for 30 minutes (Kun Shan Ultrasonic Instruments, Jiangsu, China) for 30 minutes. The mixture was centrifuged for 10 minutes at 1180 × g (Sigma, St. Louis, MO, USA). The precipitate was collected, washed with ethanol, dissolved in 50 mL distilled water, and heated for 2 hours. The supernatant was transferred to a 100 mL volumetric flask and diluted with water to 100 mL. The iodine test, which is used to determine the presence of starch or dextrin, was applied. No color change was observed, indicating that starch and dextrin were not present in the test solution. Next, the polysaccharide content in the test solution was assessed using the phenol–sulfuric acid method, following the basic protocol specified by Dubois et al. Crude polysaccharide dissolution can be determined using the following equation:

\[
\omega = \frac{m_1 \times V_1}{m_2 \times V_2} \times 0.9 \times 10^{-4}
\]

where \(m_1\) (μg) is the crude polysaccharide content of the sample solution, \(V_1\) (mL) is the constant volume of the sample solution, \(V_2\) (mL) is the test volume of the sample solution, \(m_2\) (g) is the weight of C. sinensis, and 0.9 is the correction factor for the conversion of glucose into dextran.

Statistical analysis

Data were expressed as the mean (SD) of a minimum of three replicates independent experiments. The Student’s t-test was used to compare treatment groups, using SAS version 9.3 for Windows (SAS Institute Inc., USA). The level of statistical significance was set at \(P < .05\).

Results

Effect of heat treatment on SOD activity in C. sinensis

Generally, enzymes are heat-labile, easy to deactivate and denature. However, in this study, heat treatment did not decrease SOD activity (Fig. 2). On the contrary, most of the
heat treatment stimulated the enzyme activity except for the group treated for 60 minutes at 60°C stimulated the enzyme activity. SOD activity of C. sinensis treated for 30 minutes at 80°C was significantly higher (61.43%) than that of the control (P < .05).

**Effect of heat treatment on DNase activity in C. sinensis**

The DNase activity of the C. sinensis aqueous extract was measured using the plasmid-nicking assay. Shorter heat treatments of 15 or 30 minutes treatment at all three temperatures appeared to stimulate DNase activity, although this was not statistically significant when compared with the DNase activity of the untreated group (P > .05). Data are presented in Fig. 3.

**Effect of heat treatment on the dissolution of cordycepin**

Cordycepin, a nucleoside analog, is one of the main active components in Cordyceps in C. sinensis. It is able to withstand 24 hours of 95°C hot water extraction, which suggests that it is not denatured by heat treatment. However, heat treatment might affect its dissolution. Therefore, we measured changes in cordycepin dissolution following heat treatment (Fig. 4). Interestingly, cordycepin dissolution increased slightly in the heat-treatment groups. In particular, it was significantly higher in the groups treated at 60°C for 60 min and at 80°C for 15 minutes (P < .05) than in the control group.

**Effect of heat treatment on polysaccharide dissolution**

Polysaccharide dissolution was measured after various heat treatments; data are presented in Fig. 5. There was no significant difference between the control and heat-treated groups (P > .05).

**Discussion**

In this study, heat treatment increased the dissolution of cordycepin and polysaccharide which is benefit for their oral bioavailability. The heat treatment has no adverse effects on the DNase and SOD activity of C. sinensis. Several components of wild C. sinensis have been found to be related to its various pharmacological activities; of these, cordycepin (3’-deoxyadenosine) and polysaccharides are specific components. Nucleosides are believed to be one of the key active components of wild C. sinensis. Cordycepin contributes to the anti-tumor, insecticidal, and antibacterial activity of C. sinensis and is used as a marker for quality control. Polysaccharides are one of the most abundant components of the fungus and a major group of bioactive constituents; they have been extracted and isolated from the fruiting bodies, cultured mycelium, and fermentation broth, and are structurally diverse biomacromolecules with varying physiochemical properties. They contribute to the anti-inflammatory, antioxidant, anti-tumor, anti-metastatic, immunomodulatory, anti-diabetic, steroidogenic and hypolipidemic effects of the wild C. sinensis, and they have been the target of the development and quality control of wild C. sinensis health products.

The activity of proteins, particularly enzymes, is generally not stable to heat treatment. Very few C. sinensis proteins were investigated despite the total protein content of C. sinensis being approximately 23.5–35.8% by weight. SOD is an important active protein that contributes to the antioxidant activity of C. sinensis. One gram of total protein contains approximately 65 mg of SOD. DNase is another enzyme that has been identified in C. sinensis.

![Figure 3](image-url)  
**Figure 3**  
Effect of heat treatment on deoxyribonuclease (DNase) activity in Cordyceps sinensis (mean (SD), n = 3).  
*Significantly different from the control (P < .05).
DNase is widely present in bacteria, fungi, plants, and other lower organisms and higher vertebrates. It was the first acid DNase to be identified in a fungus. Therefore, SOD and DNase enzyme activity were chosen as indexes to evaluate the effect of heat treatment on enzyme activities in wild *C. sinensis*.

The effects of heat treatment on SOD and DNase activity and the dissolution of polysaccharides and cordycepin in *C. sinensis* have been summarized in Table 1. The data indicated that heat treatment has no adverse effects on the biological activity of *C. sinensis* or the dissolution of its bioactive components. In fact, heat treatment stimulated enzyme activity to some extent—for example, treatment at 80°C for 30 minutes increased SOD activity. Treatment at 100°C for 60 minutes does not adversely affect the activity of any of the bioactive components studied, and therefore this treatment paradigm is recommended before packaging in order to destroy bacterial, viral, and insect contaminants and prolong the storage time of *C. sinensis*.

This study also examined the DNase activity of wild *C. sinensis*. It is notable that wild *C. sinensis* DNase retained its activity after 60 minutes of heat treatment at 100°C, which abolishes DNase activity in several other organisms. For example, pancreatic bovine pancreatic deoxyribonuclease I (bpDNase I), which is a widely used enzyme in molecular biology, loses activity quickly at 68°C (the rate of inactivation is too rapid to measure). In addition, a new acid deoxyribonuclease (DNase) was purified from the cultured mycelia of *C. sinensis*. However, this enzyme completely lost its activity after 15 minutes at 80°C. We offer two possible explanations for the seemingly contradictory response of DNase activity to heat treatment.
observed in the present study. First, these DNases in *C. sinensis* might be heat-stable ones. It is possible that new heat-stable DNases is existed in *C. sinensis*. In the present study, the DNases in wild *C. sinensis* were in a natural protective environment, rather than in the purified state, which might be another explanation for its superior heat stability.

The discovery of the enzymatic activity of SOD purified from bovine blood was first reported in 1969 by McCord and Fridovich,23 and it was subsequently isolated from bacteria, fungi, algae, plants, insects, and several other organisms. However, the enzyme activity of SOD in wild *C. sinensis* is reported for the first time in the present study. SOD, as a globulin with high thermal stability, demonstrated no loss of activity in response to treatment at 80°C for 30 minutes. For example, Cu/Zn-SOD enzyme, isolated from *Meretrix meretrix* remains relatively stable at temperatures below 50°C, at which more than 83% of the activity was maintained. At temperature above 50°C, the enzyme becomes unstable, with complete loss of activity after incubation at 90°C for 10 minutes.24 The activity of EC-SOD (extracellular superoxide dismutase, which exits extensively in every living creature like animal, plant, and microorganism) enzyme was maintained at 100% at 60°C for 60 minutes; however, it is inactivated abruptly when the temperature is increased to 80°C.25 In addition, 20% of the activity of SOD purified from *Cordyceps militaris* was maintained at when exposed to a temperature of 90°C for 20 minutes.26 It is, however, remarkable that the activity of wild *C. sinensis* SOD is retained after treatment for 60 minutes at 100°C. It is possible that the enzymes in wild *C. sinensis* are unique in terms of heat stability because the fungus grows on the Tibetan plateau, a region with extreme weather conditions.

Overall, heat treatment had no adverse effects on the assessed bioactive components of wild *C. sinensis*; it is an environmentally friendly and low-cost technology that is simple to use and poses little or no risk to users and bystanders.27 Therefore, heat treatment might be an appropriate means to extend the shelf life of wild *C. sinensis*.

### Conclusion

Heat treatment for less than 60 minutes at below 100°C did not decrease the activity of SOD and DNase from wild *C. sinensis*, and the dissolution of polysaccharides and cordycepin were not affected by this treatment paradigm. These results suggested that heat treatment might be a safe processing method to extend the storage time of wild *C. sinensis* without loss of biological activity.

### Competing interest

We declare that we do not have any commercial or associative interests that conflict with the work submitted.

### Authors’ contributions

PKW, ZT, and CMW conceived and designed the study. PKW, ZT, HFL, GXJ, and DG performed the experiments. CHM authenticated the identification of the *Cordyceps sinensis* specimens used in this research. PKW and CMW wrote the manuscript. All authors read and approved the final version of the manuscript.

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